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Plant Pathogenic Bacteria

Versailles (France)
June 9-12, 1992

M. LEMATTRE, S. FREIGOUN,
K. RUDOLPH & J.G. SWINGS
Editors

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Ladies and gentlemen, dear colleagues,

The Organizing Committee feels it a great privilege to welcome you at the 8th International Conference on Plant Pathogenic Bacteria in Versailles.
I would express my melancholy for the absence of Professor Kelman and Professor Schroth who could not attend the Conference.
Some weeks ago, I heard with sadness that our dear friend and colleague Dr Maria de Lourdes de Oliveira passed away this year. She was a famous scientist in Bacteriology and everybody remembers the successful Conference that she organized in Oeras with her husband. Her contribution enriched the science and she will stay in our memory like a model for each of us.
Since 1964 at Harpenden, Conference after Conference, our common interest for Phytobacteriology meet us and establish friendly contacts and like in a family, the first generation discover with sympathetic but critical attention the new generation's point of view and approaches.
In Versailles,during four days, 246 scientists from 43 countries will present their contributions. According to the wishes of ISPP Committee we proposed, ten years after the Conference in Cali, to examine with particular attention the advances in research on tropical diseases and discuss together the new approaches and the scientific means to control these diseases which are actually discriminative for the economy of numerous countries in tropical areas.
For the first time this Conference extends its interest to different procaryotes BLO and MLO which will enrich the discussions.
An important contribution of 231 abstracts has been submitted to the Committee. To increase the exchanges we suggested to present in a single room all the oral communications and the different syntheses of posters contributions.
The choice has been difficult to realize this programme in four days.
I thank the members of Organizing Committee for their efficient participation and session leaders who accepted the difficult work to link this very large information and who will help us in discussions and realization of successful projects.
If pathogenicity and detection sessions are particularly compact, we regret a limited participation in the very interesting but difficult research area of epidemiology and control. We hope however that a fruitful work will be realized.
In this programme we have not forget workshop activities : four groups asked us to organize with their chairman round tables or meetings.
Many symposiums on *Pseudomonas agrobacterium, Erwinia amylovora*, and the famous Seatle Congress limited your participation and I think that in the future we must better integrate these different activities in our projects.
Let me introduce now Dr A. Coleno, who accepted to present, his point of view on in Plant Bacteriology research. He has worked many years as a plant bacterologist although he recently left for an administrative position.
I wish you a both fruitful and enjoyable Conference in Versailles.

Monique LEMATTRE

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Special appreciation is also extended to A. Barazer, G. Lacaze, M. Kalka for typing the Conference Programme and Abstracts. The assistance of Researchers and Students of Plant Pathology Station contributed to the success of the Conference and was especially appreciated by the Organizing Committee.

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Introduction

New Trends in Phytobacteriology

First, I want to say how much I am pleased to be here at the opening session of the VIIIth Conference of Plant Pathogenic bacteria. I thank Madame LEMATTRE to give me the opportunity to say some words at this occasion.
Normally as a French official representative it would be better to give my speech in French but as ISPP secretary it would be better to give it in English. So I will speak in French but for I own funny device which automatically translates French into English. I put it on the microphone. The only problem is the translation is usually not as good as you could expect. So be sure my French is correct and any mistakes will denote a translation problem.

Well, what about phytobacteriology in the next future ? When we look at the program of the VIIIth Conference, it appears a real permanence of the objectives :

1 - phytobacteriologists now are still working on characterisation of plant pathogens aspects and on etiology. 18 papers are on these subjects.
27 on taxonomy. very natural and encouraging. We need to know well the organisms we are working on.

2 - Diagnostic and detection are also a constant preoccupation. 40 papers present results of modern approaches for quick and specific detection inside plants or on plant surfaces, in weeds, in soil and rhizosphere. The aim of these works is to get the certitude that a sample could or could not be declared safe, which is fundamental on a quarantine aspect. Seems to me there are not enough effort on the evolution of the inoculum, in terms of quality and quantity, in terms of potentiality regards to the plant population in agronomical situation.

3 - Interaction between plant and bacterial pathogens that includes all works on pathogenic determinants and reaction of plants is undoubtally a focal objective. The use of molecular approaches considerally enhanced the studies, 70 papers are on.
It is evident that these approaches will be maintained and developed in the future. It is the only way to understand precisely the chronology of the events in a plant parasite relationships, to know why a plant pathogenic bacteria is pathogenic.

4 - The two other important topics in this conference are ecology, 35 papers, and biocontrol (including PGPR), 37 papers. Hopefull subjects. We know bacterial plant pathogens are responsible of important decay. It will be very useful to get a safe and credible biocontrol. Independently with the fact that there is some shadow on the use of genetic engineering microorganisms (but this is another question), I am sure studies on biocontrol and ecology will be developed.

5 - But I am surprised to see so little work or interest on evolution of phytobacteria. I remember PALLERONI and DOUDOROFF's statement in 72 in annual review of phytopathology about the protection importance of phytopathogenic bacteria and more precisely pseudomonas for studying speciation. With the genetics markers we have now and with an approach of evolutionary genetics bacterial plant pathogens remain such good candidates.

In the same sense «resistance of plants» also seems to be a little forsake. With the results obtained on the plant parasite interaction at the cellular level, this will change. The way we study now plant parasite interaction is very exciting, but we have to remember that our main goal is to offer a control system which is economic, safe, easy to use for each disease you work on and it is what I wish for you.

Alain COLENO
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des Productions Végétales (1)

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I

Evolution of phytopathogenic bacterial problems. Particular aspects of tropical bacterial diseases

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Advances of research on bacterial blight of rice *(Xanthomonas oryzae* pv. *oryzae)*

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It is a great pleasure and an honour to present this invited paper in the Introductory Session. We wish to report the progress made over the last few years in research on bacterial blight of rice. The disease is caused by *Xanthomonas oryzae* pv.*oryzae* and was first reported in Japan in 1884. Among the eight bacterial diseases of rice in tropical Asia, bacterial blight has received most of the attention because of its high epidemic potential and destruction to rice. Our experience working with bacterial blight involves a network of scientists from both developing and developed countries. Our research was initiated to address breeding for varietal resistance as a means to control the disease. Despite some progress made the disease continues to pose a threat to rice production in tropical Asia. What other options do we have? How can we capitalize on advancements of molecular biology to serve our purposes?

The Bacterial Diseases of Rice

In tropical Asia, there are about eight bacterial diseases of rice. Each can cause damage to the rice plant and induce serious yield losses under specific ecosystems during the monsoon season. Among them, bacterial blight caused by *Xanthomonas oryzae* pv.*oryzae* has received most of research attention because of its worldwide distribution, and destructiveness in the rainy season when most of the rice is grown. Bacterial blight has now become endemic on rice in many countries. Reductions in rice yield have not been well assessed however, partly due to the fact that we have little understanding of the epidemiology of the disease in the tropics.

While both bacterial blight and bacterial streak [renamed as pathovars of *Xanthomonas oryzae, X. o.* pv. *oryzae and X. o.* pv. *oryzicola* (Swings et al., 1991)] have been studied intensively, the other bacterial diseases of rice caused by *Pseudomonas* spp. and *Erwinia* spp. have received very little attention until now. A recent survey has shown that there are a few pseudomonad pathogens found associated with seed rot and sheath discoloration in the wet season in the Philippines (F. Van Otryve, T. W. Mew & J. Swings, unpublished data), and perhaps also in other tropical Asian countries. A disease which is known as "red stripe" has caused serious damage to rice in Indonesia and Vietnam in recent years. A pseudomonad pathogen was isolated from the infected leaf tissues. However, since Koch's postulates have not been fulfilled to date, the causal organism has not yet been fully established for this increasingly important disease. This information suggests that while we are successful in keeping one disease in check, others have emerged. In this presentation, we shall focus only on bacterial blight.

To Understand The Pathogen Variation

There were several epidemics of bacterial blight reported in the 60's and 70's (Mew, 1987) when the modern semi-dwarf, nitrogen responsive cultivars were developed and introduced. There was little effort to control it through means of cultural management. Varietal resistance is considered most effective and economic. The first modern rice cultivar developed through cross breeding for bacterial resistance is IR 20. But like any other host resistance to plant diseases, new races were detected to cause breakdown of the resistance almost immediately after its release to farmers. When we initiated our project in mid-70s, the first question we asked was "how variable is the bacterial blight pathogen?" It is obvious that effective varietal resistance depends closely on understanding of the pathogenic variability in response to the host resistance in rice growing ecosystems.

We started by collecting isolates from both improved semi-dwarf rice cultivars and from traditional farmer's cultivars all over the Philippines, and tested their virulence on hundreds and thousands of rice germplasm accessions conserved in IRRI's genebank. Then we collated and evaluate cultivars known to differ in resistance (Mew, 1987). Our

objective was to assess if there was differential interaction between *Oryza sativa* and *X. oryzae* in the tropics where rice was continuously cultivated over centuries. With a set of four rice cultivars, IR 8 (known at that time to carry no functional resistance to bacterial blight in the Philippines); IR 20 (with a dominant resistance gene *Xa-4*); and IR 1545-339 (with a recessive resistance gene *xa-5*), we were able to recognise four bacterial groups, later known as four races. The differential interaction was confined to a specific cultivar-strain combination. The differential interaction between *Oryza sativa* and *X. oryzae* in the tropics was thus demonstrated.

Using this set of differential cultivars, later including CAS 209 with the *Xa-10* gene, and DV 85 with the resistance genes *xa-5* and *Xa-7*, the distribution and frequency of *X. o.* pv. *oryzae* races was monitored over the past 20 years. There was an apparent shift in frequency of race distribution of the bacterial population after wide cultivation of modern cultivars with the *Xa-4* gene (Mew et al., 1992). The predominance of race 2 may be due to the greater compatibility of this race with currently grown cultivars possessing the *Xa-4* gene. Strains of race 2 appear more aggressive on cultivars with the *Xa-4* gene. But race 1 appeared better adapted than race 2 in traditional cultivars or cultivars not carrying the *Xa-4* gene. Thus, a readjustment or rearrangement of the original population of races in response to cultivation of cultivars possessing the *Xa-4* gene has occurred. Whether the race situation will change, and how rapidly it will change, obviously depends the deployment of other rice cultivars with different resistance genes. This is an area of research we are currently engaged in.

Information obtained from monitoring the race distribution has indicated geographic structuring of the pathogen population within Philippines. Generally, we may divide the races into two ecotypes. It appears that there is a transition zone on the actual distribution of the two ecotypes. Data has indicated that there is a shift of one race group from high altitude to a transition zone where mixture of high-land races and lowland races were observed then to the lowland area with lowland race. For instance, all strains collected from mountain provinces about 5000 ft above sea level of the Philippines have

been identified as race 5 on the differential rice cultivars. Race 5 represents a high land race group while other races such as races 1, 2, 3, 4, etc. belong to the lowland race group. Although there is no experimental data to suggest what environmental conditions may influence their distribution, temperature probably plays an important role.

Advances in recombinant DNA technology have provided a powerful tool to study the structure of the pathogen population. The information gained will not only enhance our understanding of the pathogen on the population level, but also provide insight into the way in which pathogen populations may respond when a resistance cultivar is deployed. Initially we focused on pathogen population structure in the Philippines. In collaboration with Jan E. Leach of Kansas State University, and Hei Leung of Washington State University, we have applied DNA typing of *X. o.* pv. *oryzae* races to refine the monitoring of race distribution and pathogen organization in the Philippines.

The highly repetitive DNA element pJEL101 was isolated from *X. o. oryzae* (Leach *et al.*, 1990). This element was later shown to have the properties of a transposable element, and was designated IS203 (Yun, 1991). IS203 was used for DNA fingerprinting of a collection of *X. o. oryzae* strains from the Philippines (Leach et al., 1992). The DNA profiles obtained from 97 strains were subjected to numerical analysis, to obtain a dendrogram representing the structure of the pathogen population. The population was found to consist of five genetic lineages at the 85% similarity level. There was a somewhat complex relationship between lineages and pathotypes.

To determine whether the inferred diversity and phylogeny of the *X. o. oryzae* population was dependent on the probe used, we analyzed a similar collection of strains with multiple probes (Nelson et al., in preparation). Using a transposon trapping vector (Kearney and Staskawicz, 1990), four distinct transposable elements were isolated from the genome of *X. o. oryzae.* Our results suggested that different probes give rather different information on the pathogen population. Not all probes were equally informative in revealing genetic variation among strains. The estimates of diversity for the different races were highly dependent on the probe used.

In addition to the transposable element probes, an avirulence gene was also used as a probe for RFLP analysis of the same set of isolates (Vera Cruz and Leach, pers. comm.). An avirulence gene is one that is involved in determining the cultivar specificity of the pathogen strain. The gene avrXa10 was cloned from *X. oryzae* by Hopkins *et al.* (1992). This clone hybridized to a family of genes, including other avirulence genes. We had expected that this probe might be better than the transposable element probes for distinguishing strains with different pathogenic specializations. The dendrogram derived from the avirulence gene probe was, however, very similar to that derived from the other probes. The results confirmed the phylogenetic relationships inferred from the other probes, and suggested that the molecular differences between alleles of avrXa10 are not necessarily distinguishable by simple Southern blot analysis.

While the tree topologies derived based on the RFLP data obtained using the different probes were somewhat different, a basic view of the population structure of the pathogen was derived from this analysis. Four main pathogen lineages were identified, based on the consensus among the datasets. Two of the lineages consisted of multiple pathogen races, and three of the six Philippine races were found to be composed of two distinct lineages each.

Groups of strains representing the pairs of lineages from each race were tested on cultivars carrying known resistance genes. Differential reactions were observed and confirmed for each race. Thus, three new races of the pathogen have been detected, corresponding to pathogen lineages detected through DNA typing.

Based on RFLP typing, and on historical and pathotypic information, a picture of *X. oryzae* race evolution in the Philippines was pieced together. Race 1 was dominant in the lowland areas of the Luzon, Philippines until cultivars carrying *Xa*-4 were widely deployed (Mew et al., 1992). In response to selection exerted by Xa-4, race 2 (compatible with *Xa*-4) became the dominant race (Mew *et al.*, 1992). Race 2 was apparently derived not from race 1, however, but rather from a lineage dominant in the highland areas of Luzon. Race 3, also compatible with *Xa*-4 and apparently derived from race 1, has also established itself in areas of Luzon.

In collaboration with scientists in national programs, we aim at understanding the lineages of *X. o.* pv. *oryzae* in Asia, and their spectra of virulence relative to known resistance genes and to indigenous rice cultivars. A practical goal is to develop cultivars with durable resistance based on information on pathogen and host genetics. To complement resistance breeding, knowledge of the pathosystem can be used to design deployment strategies to maximize the useful lifetimes of resistant varieties. To develop such deployment strategies, knowledge of spatial and temporal distribution of the pathogen is needed. One such strategy for slowing the evolution of compatible races involves the use of varietal heterogeneity.

Simplified marker techniques would allow more efficient handling of large samples sizes. Digestion of DNA extracted from strains of *X. o.* pv. *oryzae* with the restriction enzyme *Pst*I produced distinctive restriction patterns for different strains using conventional agarose gel electrophoresis (Raymundo et al., 1992). While similar groupings of strains were defined based on RFLP (pJEL101 and TNX1) analysis and by *Pst*I digestion, *Pst*I typing is rapid and less expensive.

At present, nine races have been identified in the Philippines based on reactions with the IRRI differential cultivars which contain resistance genes, namely IR24 (0), IR20 *(Xa-4)*, Cas 209 *(Xa-10)*, IR1545-339 (*xa-5*), and DV85 (*xa-5, Xa-7*). Using these and other differential cultivars, many more races may be identified and described from other geographic areas. However, not all the resistance genes are functional across a diverse rice ecosystems ranging from tropical irrigated to temperate irrigated ecology, from rainfed to rainfed lowland, from deepwater to tidal wetland ecosystems. Virulence of *X. o.* pv. *oryzae* must have evolved together with *Oryza sativa* over thousands of years. Crop intensity together with crop environment should have a strong effect on the pathogenic variability and diversity. The problems related to use varietal resistance for disease management are often associated to how durable is the resistance conferred by the resistance gene(s). What impact of a host genotype grown under specific agro-climatic zones, and cropping intensity on variability of *X. o. oryzae* is a complex issue to assess but we need an answer, so the

genetic resources can be intelligently utilized and perhaps re-cycled. With all the progress made and tools, especially molecular tools available in the rice-*X. oryzae* pv. *oryzae* system, it appears we can now address this question with confidence.

To Understand Host Resistance

Bacterial blight has been effectively controlled by planting resistant cultivars. The first modern rice cultivar developed through cross breeding for bacterial blight resistance is IR 20. A new race able to breakdown the resistance was detected almost immediately after its release to farmers.

So far, 19 resistance genes have been identified (Ogawa and Khush, 1989; Kinoshita, 1991). Strong differential reactions between rice cultivars and the races indicate qualitative resistance. The 19 resistance genes apparently confer the resistance of this type. Genes for resistance to bacterial blight were evaluated against different races and sets of near-isogenic lines (NILs) have been developed (Ogawa and Yamamoto, 1987; Ogawa et al., 1990). NILs were produced using three different recurrent parents: Toyonishiki, a japonica rice; Milyang 23, an indica-japonica hybrid; and IR 24, an indica. We are interested in the the effect of genotype-environment interaction on the expression of resistance, and the effect of the genetic background on the expression of resistance. Seeds are now available for race testing and comparison by scientists in different rice growing countries. Single gene resistance to bacterial blight can also be evaluated in different bacterial blight hot spots.

Initial testing at IRRI suggests that, in addition to major genes, there are quantitative resistance genes with race specific effect. The gene *Xa-14* shows qualitative resistance to incompatible race, but its reaction to compatible race, there was significant difference in lesion length caused by different strains of the same race. We would like to know more about genes with quantitative effects on disease expression, and to know whether some such genes might be race non-specific.

Different lesion types of bacterial blight are observed when testing the isogenic lines against incompatible races. Three lesion types were recognised with the incompatible reactions on the differential cultivars of the isogenic lines using the Philippine races (J. F. Bai and T. W. Mew unpublished data). These lesion types also known as infection types are necrotic, chlorotic lesions and asymptomatic type. Lines with *Xa-3* gene in response to the infection of an incompatible race produce a necrotic lesion, while *Xa-4*, *Xa-5*, *xa-5* and *Xa-7*, a chlorotic lesion. Lines with the *Xa-10* produce asymptomatic lesion. Kaku and Kimura (1987) also showed distinct lesion type associated to genes of resistance against the Japanese races. Further characterization of resistance of the isogenic lines with three recurrent background is on progress.

The advancement of molecular genetics and genomic mapping of rice plant will facilitate selection efficiency. We have applied molecular markers and gene tagging to understand the genetics of bacterial blight resistance and to assess different gene combinations. In collaboration with S. Tanksley of Cornell University and A. Yoshimura of Kyushu University, several of these genes have been mapped on the rice genome and tagged using molecular markers (McCouch et al., 1991; Ronald and Tanksley, 1991; Yoshimura et al. 1992). These mapping efforts have taken advantage of the availability of the near isogenic lines.

DNA markers linked to resistance genes will help in the selection of lines carrying different gene combinations, particularly when the presence of one gene obscures the action of the other gene(s) to be selected. Lines carrying *Xa-4* + *xa-5* and *Xa-4* + *Xa-10* were selected using RFLP markers linked to these *Xa* genes, together with pathogen inoculations when possible. The lines carrying pairs of genes were tested against compatible and incompatible races to the respective single genes. Complementary gene interactions were observed for reaction to race 4 of the bacterial blight pathogen. Lines carrying the *Xa-4* + *xa-5* and *Xa-4* + *Xa-10* were more resistant to race 4 than either of the parental lines which was susceptible.

The ability to characterize and understand pathogen populations with greater detail will enhance rice varietal improvement and deployment strategies. The problems related to use varietal resistance for disease management are often associated to lack of understanding as the resistance genes to be used, and the durability of the resistance conferred by the resistance gene(s), and the ecosystems where the improved rice cultivar is deployed. With all the progress made and availability of molecular tools in the rice-*X. o.* pv. *oryzae* system, we can begin to understand the impacts of the host genotypes under specific agro-climatic zones, and cropping intensity on the variability of *X. o.* pv. *oryzae*.

To Understand the Disease in the Rice Ecosystems

Our research on bacterial blight is designed around the disease triangular relationship of the host-pathogen-environment. The weakest link of our research is the understanding of its epidemiology. In the literatures (Mizukami and Wakimoto, 1969), although there are adequate informations describing the conditions conducive to the disease development and spread, yet there is no adequate quantitative data in relation of pathogen population establishment relative to the host population in defined ecosystems. We have not adequately characterized the disease epidemics in the diverse rice growing environments. The input use in rice production varies from region to region and country to country. And we have little information as the level of rice production input on the disease epidemic. The deficiency may be partly related to lack of information on the influence of bacterial blight infection on host physiology, and partly because the diagnostic tools which enable us to quantify the pathogen build-up has not been available. Although the bacteriophage technique has been used extensively in the study of bacterial blight epidemic, variation between bacteriophage and the host bacterial strains has limited its usefulness. With the development of monoclonal antibodies (MAbs) and DNA probes against *X. o.* pv. *oryzae*, new tools are now available for both detection, identification, and establishing the relationship between races and host genotypes under specific environment where the disease may become epidemic. For spatial and temporal distribution, monitoring of the pathogen with MAbs may allow for rapid identification of wild type and

can be used in simple assays such as ELISA. Thus, with the availability of MAbs which make analysis of a large number of samples is required, both phenotypic and genotypic epidemiological studies of bacterial blight is possible.

In collaboration with Ann Alvarez, University of Hawaii, monoclonal antibiodies against *X. o.* pv. *oryzae* have been developed (Benedict et al., 1989). We have applied MAbs to map the distributions of two strains, PXO61 (race 1) and PXO86 (race 2) and monitor the disease progress under field conditions in the Philippines. As a second marker, clones selected for antibiotic resistance were employed to facilitate their recovery on semiselective media. At the termination of two field trials, the race 1 was predominant in all plots and appeared to have greater epidemic potential on a rice cultivar which lacked genes for resistance. To complement these studies, further analysis based on RFLP patterns of a smaller subset of the strains recovered from field samples is in progress.

To Strengthen International Collaboration

The rapid progress in the research of *X. o.* pv. *oryzae* has made possible through close international collaboration between scientists from developing and developed countries. We anticipate this collaborative network to continue to solve the basic and applied problems of bacterial blight. A summary of progress and new challenges have recently been presented in a Feature Article published in Plant Disease (Mew et al., 1992). We believe the intensive international collaboration, and the application of powerful tools advanced from molecular genetics and biology enable us to address questions which were not possible in previous years. Despite the advances gained so far, our objective remains solving a downstream problem with the upstream research. It is a team's effort that enables progress made in a short spin of time. When resource for research is getting scarce and when losses due to plant diseases may affect the immediate livelihood of millions of people, the collaborative approach has set to solve an important rice disease threatening rice production in tropical Asia.

I recall in the 7th Conference held at Budapest, Arthur Kelman pleaded that in plant pathology we need a few *E. coli* to foster our research effort to advance our science.

Although we are not sure whether *Xanthomonas oryzae* will become one of plant pathologists' *E. coli,* the problem on the crop - rice it causes, is serious enough and we have to focus our research to provide solution to management the disease for better crop production.

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The life and times of *Pseudomonas solanacearum*

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ABSTRACT

There is increasing evidence that P. solanacearum is an ancient, homogenous species that is only remotely related to other bacterial plant pathogens. Strains of this bacterium appear to be the product of long evolution that has occurred independently at widely separate geographic locations. Analysis of DNA restriction fragment polymorphisms (RFLP) indicates that there are large clonal populations of P. solanacearum in different regions of the world. From a study of more than 200 strains, it is apparent that, early in evolution, the bacterium became divided into two geographically distinct populations, now represented by numerous strains that developed at isolated locations in the New World and the Old World (COOK & SEQUEIRA, 1989; COOK et al, 1991). Within each division, many of the RFLP groups consist of large clonal groups of strains that attack several hosts in extensive geographic areas, while other groups involve relatively few strains that attack specific hosts at restricted locations. In this paper, I have stressed: a) cases of parallel evolution of the bacterium on species of Musa in Southeast Asia and on Heliconia in the Caribbean region, which led to the development of strains (represented by distinct RFLP groups) that are highly pathogenic on banana and plantain, b) RFLP data that suggest that all race 3 strains from potato have a common center of origin in the Andean highlands and that humans were responsible for the dissemination of these strains throughout the world.

Introduction

Bacterial wilt caused by Pseudomonas solanacearum is an important disease of many crop plants in tropical and warm-temperate regions of the world. In spite of extensive research on this bacterium for over a century, the economic impact of the disease, particularly in developing countries, continues to increase. To a large extent, this is the result of the existence of a large number of strains with ever-expanding host ranges that now include several important tree species that only a decade

ago were thought to be resistant to bacterial wilt. These strains have been classified into five races, according to host range and geographic origin, or five biovars, according to biochemical characteristics (HAYWARD, 1964, 1991; BUDDENHAGEN & KELMAN, 1964).

The purpose of this paper is to summarize what we know about the taxonomic relationships of this organism, as revealed by modern techniques of molecular biology, and to present some notions as to how it has evolved and how new strains may have developed. Much of the work discussed here is the result of research completed by my collaborators, Douglas Cook and Elizabeth Barlow. In addition, I have made liberal use of the literature on the biology of this organism, which has been summarized in excellent, recent reviews (HAYWARD, 1991; BUDDENHAGEN, 1985)

Phylogeny

There is clear evidence that P. solanacearum is quite distinct taxonomically from other plant pathogenic pseudomonads as well as from members of the closely-related genus, Xanthomonas. This was established early on the basis of numerical analysis of phenotypic traits (COLWELL & LISTON, 1961), DNA/DNA hybridization (PALLERONI et al, 1973), and RNA/DNA hybridization (DE VOS et al, 1985). Among the many peculiarities of P. solanacearum are the facts that: a) it exports important extracellular enzymes (e.g. endoglucanase) via a two-step process involving a lipopoprotein intermediate (HUANG & SCHELL, 1990b), b) it has unusual translational start codons (e.g. TTG) for certain genes (HUANG & SCHELL, 1990a), and c) there are unusual nucleotide sequences in its 16S ribosomal RNA, which lead to a peculiar secondary structure (STACKENBRANDT et al, 1988). The general consensus is that P. solanacearum is a phylogenetically distinct, homogeneous group that is only distantly related to other homology groups within the genus Pseudomonas. In support of this conclusion, recent work has determined that P. solanacearum belongs in rRNA homology group II, but that it bears little relationship to the other plant pathogens within this group. The only species that show a relationship with P. solanacearum are P. picketti and P. syzygii (RALSTON et al, 1973; ROBERTS et al, 1990). The first species is an occasional pathogen of humans, particularly in hospital situations, and the second is the cause of a wilt disease of clove in Sumatra.

Origin and Geographic Range

Most of the available evidence suggests that P. solanacearum is an ancient species that arose early in geological history, possibly as a pathogen of the ancestors of modern plants. The facts that: a) this bacterium occurs as numerous strains with distinct host ranges in many parts of the world, and b) it is found in virgin soils, attacking native plants in the forests of Central America, Florida, and Indonesia suggest that it has been present in tropical areas for eons (BUDDENHAGEN & KELMAN, 1964). We do not know, of course, whether this organism originated only once at a particular location in one continent, later spreading to other continents, or whether it evolved separately in many different locations. We do know that man is a very recent interloper who has contributed significantly to the expansion of the geographic range of this bacterium.

In 1964, BUDDENHAGEN & KELMAN concluded that strains of P. solanacearum are the product of long evolution that has occurred independently in various areas and on different hosts. The evidence from modern methods of genetic analysis, and discussed in this paper, would confirm that view. Thus, the hypothesis that under the selective pressure of the soil-root environment some soil pseudomonad is constantly being converted into new strains of P. solanacearum is most improbable.

Variability in Pseudomonas solanacearum

The large number of strains of P. solanacearum that exist worlwide presents difficult problems for the taxonomist as well as for extension and quarantine personnel who need to establish criteria for differentiation of strains. The binary system of classification (five biovars and five races) presently in use is confusing because each biovar contains strains with different host ranges and host range transects the biovar system (COOK et al, 1991). The race system indicates that there are natural groupings based on host range. For example, race 3, a pathogen mostly of potato from the Andean highlands, is composed exclusively of strains that belong in biovar 2 and has phenotypic characteristics that are readily identifiable. On the other hand, race 1 contains a wide diversity of strains and encompasses numerous biovars. Thus, the traditional methods of classification do not account for the natural variability in P. solanacearum.

RFLP Analysis of Strains of Pseudomonas solanacearum

A few years ago, we began an analysis of restriction fragment polymorphism (RFLP) in strains of P. solanacearum with the use of nine DNA probes, seven of which encode information essential for virulence and the hypersensitive response (COOK & SEQUEIRA, 1989). These studies established the existence of 28 distinct RFLP groups in an initial sample of 62 strains. The work has now been extended to over 200 strains of P. solanacearum from a worldwide range of hosts and geographical locations (COOK et al, unpublished data). The number of RFLP groups was increased by only seven, indicating that the initial groupings had accounted for most of the natural variability in the species.

An important general finding from these studies is that there are very large clonal populations of the bacterium that cover extensive geographical ranges in each continent and even across continents (e.g. race 3). Thus, the common notion that this bacterium is highly variable has to be modified in view of the apparent stability of many strains that have continued to spread from their centers of origin for a very long time.

Our recent analysis of a large number of strains confirmed the initial findings that there are two major divisions, with a coefficient of similarity of only 13.5% between them (COOK & SEQUEIRA, 1989). Division I contains all members of race 1, biovars 3, 4, and 5; division II contains all members of race 1, biovar 1, and races 2 and 3. Within each division, the coefficients or similarity are very high. It was immediately apparent that over 90% of the strains in Division I were from Asia and Australia, whereas 98% of those in Division II were from the Americas. This suggests, therefore, that early in evolution, P. solanacearum was divided into two groups that developed in geographic isolation in the Old and in the New Worlds.

There are numerous examples of the role of geographic isolation in the evolution of P. solanacearum. Strains from ginger and from mulberry, for example, are only found in certain parts of Asia. Strains from Heliconia (race 2) and potato (race 3) apparently originated and evolved in Latin America. The influence of humans in the spread of these strains is clearly evident from the RFLP analysis. For example, all race 3 strains that were isolated from potatoes in Australia, Sri-Lanka, and other Asian countries belonged to the same RFLP groups (26 and 27) of those strains isolated from the Andean highlands of South America.

Statistically speaking, there is no way to explain these identical DNA patterns unless the strains all originated at the same location, presumably South America which is the ancestral home of the potato. It is apparent that, since the discovery of America, humans have been moving infected potato tubers throughout the world, a process that continues even today.

In the same fashion that RFLP analysis has helped us elucidate the influence of man in the spread of certain strains of P. solanacearum from South America to Asia, it has helped us confirm cases of parallel evolution in these same regions. Race 2 strains, for example, evolved as a pathogen of species of Heliconia in the Caribbean area, as evidenced by numerous strains recovered from virgin forests in that part of the world (SEQUEIRA & AVERRE, 1961). Some of these strains began to attack bananas, plantains, and other Musa species after these new crops were introduced from Southeast Asia, causing the so-called Moko disease. Each one of the RFLP groups (24, 25, and 28) is associated with recent Moko epidemics on bananas and plantains in the Caribbean and Amazon areas. Moko disease, however, did not exist in Asia until planting material was moved from Central America into the Philippines in recent years (BUDDENHAGEN, 1985). Recently, however, an old disease of bananas in South East Asia, called blood disease, has been shown to be caused by P. solanacearum (EDEN GREEN et al, 1990). Our RFLP analyses showed that strains causing this disease are clearly distinct from any of the strains that attack bananas in Latin America. Moko disease and blood disease, therefore, represent independent evolution of P. solanacearum on members of the family Musaceae.

Resolving Taxonomic Problems

Our ability to trace the evolution of certain strains of P. solanacearum by RFLP analysis provides the means to resolve problems in the taxonomy of this organism that have vexed investigators for many years. For example, it is well known that there is a close correspondence between race 3 and biovar 2. Race 3 is indigenous to the Andean highlands where it attacks wild and cultivated potatoes. However, strains belonging to biovar 2 have also been isolated from a wide area of tropical lowlands in the Amazon basin and have been shown to be pathogenic on potato (MARTIN et al, 1981). Thus, are these lowland strains representatives of race 3? When the RFLP patterns of 25 lowland biovar 3 were examined, they were found to be clearly distinguishable

from the highland biovar 2 strains (COOK et al, 1991). Thus, race 3 constitutes a natural group that has a restricted host range in the Andean highlands, but happens to share certain biochemical properties with other large groups from the Amazon basin. These lowland strains, if one is allowed to speculate, may have been the ancestors of the highland strains.

General Conclusions

The initial results of RFLP analysis of over 200 strains of *P. solanacearum* suggest that the method may serve as a basis for a reconsideration of the taxonomy of the species. Our increased ability to trace the evolution of individual strains and to differentiate between closely related strains gives reason to hope that the new RFLP groupings have phylogenetic significance. RFLP analysis provides evidence of the evolutionary changes that have occurred in *P. solanacearum* and supports the conclusion that it is an ancient, rather homogeneous taxon. This long evolution has resulted in numerous strains with widely divergent host ranges and geographic distribution, but, overall, the species does not appear to be as highly variable as was once thought. Notwithstanding the plasticity of the species, as evidenced by the recent appearance of strains capable of attacking *Eucalyptus* and bean in Brazil, mulberry and *Casuarina* in China, etc., DNA studies presented here indicate that *P. solanacearum* is a highly clonal and remarkably stable species.

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Citrus bacterial canker disease in tropical regions

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ABSTRACT Citrus bacterial canker disease (CBCD), caused by *Xanthomonas campestris* pv. *citri* (Xcc), probably originated in Southeast Asia. Currently, the disease occurs in several citrus-growing regions around the world, especially in areas where high temperatures and rainfall occur concomitantly during the growing season. Asiatic CBCD (CBCD-A) is the most widespread form of the disease and is the only form of the disease that is known to occur in the tropics. However, pathogenic variants of Xcc occur in these areas. CBCD lesions occur on leaves, stems and fruit. Xcc survives from year to year in twig, leaf, and fruit lesions. Xcc survival in soil and in association with weeds is limited. Natural short distance spread of Xcc occurs primarily by wind-driven rain. Long distance dispersal of the pathogen is primarily via infected propagating and planting material. Regulatory and quarantine methods are designed to prevent the introduction, dissemination and establishment of the pathogen. Integrated management of CBCD is based on disease-free planting material, varietal host resistance, windbreaks, disease forecasting, removal of inoculum sources, reducing wound damage, timely application of fixed copper- and antibiotic-containing sprays, and regulatory measures including quarantines and eradication. The ecology of Xcc in tropical citrus-growing regions is not fully understood. Although CBCD-A Xcc strains around the world appear to be genetically uniform, knowledge of the extent of pathogenic variation in these strains, as well as the relationships of these to other xanthomonads associated with citrus, is limited. Improved methods of Xcc detection are needed to better understand the ecology of Xcc and epidemiology of CBCD in the tropics. Effective CBCD management strategies, as part of comprehensive citrus health programs, need to be developed in citrus-growing areas in tropical countries.

KEYWORDS: *Xanthomonas campestris pv. citri* - *Xanthomonas* - *citri* - Epidemiology - Disease Management

Citrus bacterial canker disease (CBCD), caused by *Xanthomonas campestris* pv. *citri*, probably originated in Southeast Asia in southern China, Indonesia and/or India (GOTO, 1992). The disease occurs in about 30 citrus-growing countries around the world, and is endemic in areas where high temperature and rainfall occur concomitantly during the growing season (STALL, 1988). Although several forms of CBCD are recognized (CIVEROLO, 1984; GOTO, 1993; KOIZUMI, 1985), only the Asiatic form (CBCD-A) is known to occur in tropical citrus-growing areas. However, pathogenic variants of Xcc strains associated with CBCD-A in tropical regions have been described (CIVEROLO, 1984).

The economic significance of CBCD is related directly to effects of infection and indirectly to international quarantines and national regulatory policies directed against all forms of the disease. However, accurate loss assessments are generally not available (GOTO, 1992). CBCD-A is the most economically significant form of the disease. Lesions occur on leaves, stems, twigs and fruit. Infected fruit are blemished, reducing their commercial market value. Lesions on fruit that are infected early in development may crack and these cracks can serve as entry points for secondary organisms. Also, young, developing fruit may become deformed as they mature. Severely infected fruit on susceptible hosts may abscise prematurely. Similarly, severely infected leaves, especially those with lesions on the midrib and petiole, may defoliate, resulting in reduced tree vigor (GOTO, 1992). Severe twig infection of susceptible hosts can result in terminal dieback. Severe infection of young nursery trees may also reduce tree vigor (GOTO, 1992). In some regions, as in the tropics, CBCD-A may limit the types of citrus that can be economically grown. Infected plants and propagating material from citrus-growing areas in tropical regions are important means of long-distance dissemination of Xcc to new and expanding citriculture areas in other countries.

EPIDEMIOLOGY *Symptoms* CBCD-A lesions occur on leaves, twigs, stems and fruits, forming raised necrotic spots with a coarse surface (CIVEROLO, 1984; GOTO, 1992; STALL, 1988). Incipient leaf lesions appear as translucent yellow spots between oil glands when viewed with transmitted light. Leaf, as well as fruit, lesions

appear as pinpoint slightly raised necrotic spots. Lesions enlarge, becoming distinctly raised and erumpent. As the lesions continue to enlarge, they collapse and become crater-form with raised margins and sunken centers. Margins of leaf lesions become watersoaked and appear greasy. Advanced lesions are surrounded by characteristic yellow halos. Generally, lesion size is directly related to host susceptibility. Lesions may continue to expand up to 10 mm in diameter on leaves of very susceptible hosts. Lesions are usually circular, but may enlarge irregularly, and often coalesce. Lesions finally appear corky, with a rough surface.

Pathogen Dispersal Short distance spread of Xcc within-tree and from tree to tree within groves is effected by wind-driven rain and by contaminated mechanical equipment used in grove maintenance (CIVEROLO, 1984; GOTO, 1992; STALL, 1988). The primary means of long distance dispersal of Xcc is infected or diseased propagating material such as budwood, rootstocks and budded trees (CIVEROLO, 1984). Transmission of Xcc by insects, birds and animals has not been conclusively established. Potentially, Xcc could be disseminated long distances via diseased fruit; however, there is no record of infection resulting such fruit with CBCD lesions.

Disease Cycle Xcc overwinters in lesions on leaves and shoots infected the previous autumn. When free moisture is present, bacteria ooze from lesions. Leaves and young shoots in the first spring flush are infected by Xcc from overwintering lesions through stomates, lenticels, or wounds within 6 weeks of initiation of growth. Leaves are generally most susceptible to infection via stomates for about 14-21 days after initiation of growth. Secondary infections of leaves, twigs and fruit occur throughout the spring, summer and fall depending upon the availability of susceptible tissue and climatic conditions. Fruit is susceptible to infection for about 90 days after petal fall.

Climatic Conditions Growth of Xcc in citrus tissues occurs at 6-36 C (KOIZUMI, 1985). Initial growth of Xcc *in planta* occurs immediately after ingress, even when the temperature is 6 C, allowing the pathogen to become established in host tissues. Secondary growth *in planta* begins when lesions appear and occurs at 15-36 C. However, the time between ingress of Xcc into host tissues and secondary growth is inversely related to temperature (KOIZUMI, 1985). The optimum temperature for

CBCD lesion development is about 30 C, and the maximum temperature is 35 C (KOIZUMI, 1985).

CBCD incidence is directly related to the occurrence of abundant rainfall in the spring when new growth flushes develop, and typhoons in autumn (GOTO, 1992; KOIZUMI, 1985; STALL, 1988). Generally, CBCD occurs when the average daily temperature is more than 20 C and rainfall is over 100 mm per month, or when mean daily temperature is around 30 C and the relative humidity is above 75%. Strong winds, especially at velocities above 6m/sec, facilitate dispersal of Xcc and enhance infection. Rainwater on infected foliage may contain 10^5 to 10^8 cfu/ml (GOTO, 1992). Strong winds cause injuries on leaves and twigs and facilitate watersoaking of host tissues with Xcc-containing water.

Host Resistance There is a wide range of susceptibility to Xcc infection among *Citrus* species, hybrids and cultivars from highly resistant to highly susceptible (GOTO, 1992; KOIZUMI, 1985; KOIZUMI & KUHARA, 1982; LEITE & MOHAN, 1990). The genetic basis of susceptibity/resistance to Xcc infection is not completely understood. However, susceptibility or resistance to Xcc infection under natural conditions is also affected by the effects of rootstock on tree vigor in addition to environmental conditions and by variant strains of the pathogen (KOIZUMI, 1985; LEITE & MOHAN, 1990; STALL, 1988).

Ecology In the pathogenic or parasitic life cycle phase, Xcc grows and initiates infection in susceptible citrus tissues (GOTO, 1992). In this form, Xcc also survives in holdover cankers on spring and autumn shoots and in lesions on bark and attached leaves (GOTO, 1992). Infection of leaves in late autum before trees become dormant may result in latent infections without apparent symptoms until the following spring (GOTO, 1992).

An epiphytic phase of Xcc under natural conditions has not been clearly established. Xcc can survive epiphytically for a short time during the rainy season in tropical areas following artificial infestation of leaves; however, epiphytic populations decline to below detectable levels under dry conditions (KOIZUMI, 1985). It is not clear whether epiphytic Xcc occurs as a resident or a casual. The epidemiological significance of epiphytic Xcc may be limited (GOTO, 1992; STALL,

1988).

Xcc may survive saprophytically in association with lesions on defoliated leaves, weeds, and plant debris, and in soil (GOTO, 1992; KOIZUMI, 1985; LEITE & MOHAN, 1990). In defoliated leaves, Xcc survival may be directly related to production of extracellular polysaccharide to protect the bacterial cells (GOTO, 1992). Survival of Xcc associated with plant debris is dependent upon the decomposition rate of the plant material. Survival of Xcc in the soil is dependent upon various biotic and abiotic factors. However, Xcc survival in the phylloplane, rhizoplane, and rhizoshpere of several weeds, and in the rhizoplane and rhizosphere of citrus is very limited (GOTO, 1992; LEITE & MOHAN, 1990; STALL, 1988). There is no conclusive evidence that bacteria from these sources infect citrus under natural conditions.

MANAGEMENT Management of CBCD is based on pathogen avoidance, exclusion and eradication. Where possible, resistant host varieties are used. Sanitation measures include disease-free planting material and removal of inoculum sources by pruning and defoliation, disinfestation of tools, equipment, and clothing. Windbreaks are designed to reduce pathogen spread and infection. Disease forecasting based on the amount of overwintering inoculum sources, climatic conditions and the levels of Xcc in rainwater on diseased trees are used to predict disease incidence in advance so growers can take implement appropriate measures. Cultivation practices and harvesting are avoided when trees are wet to prevent spread of Xcc. Insecticides are used to reduce insect damage. Timely application of copper-containing compounds and antibiotics can protect susceptible tissue against primary infection. Regulatory measures including quarantines and eradication procedures are designed to preclude introduction and establishement of the pathogen in CBCD-free areas.

FUTURE CONSIDERATIONS Several aspects of CBCD in tropical regions warrant additional research. CBCD does not occur in many countries or regions where environmental conditions are favorable for infection and disease development

(STALL, 1988). Moreover, CBCD epidemics occur sporadically in some areas (GOTO, 1992). A better understanding of the nature of citrus-Xcc interactions under natural conditions is needed. The ecology of Xcc in tropical citrus-growing regions, especially with respect to survival, inoculum sources, infection, and disease development, is not fully understood. This is due, at least in part, to the lack of sufficiently sensitive techniques to detect low levels of Xcc in epidemiologically significant (micro)habitats. The relationships between Xcc and other xanthomonads associated with citrus also need to be clarified. Pathogenic variation in Xcc strains in tropical regions is poorly understood. The nature, extent and role of the interactions between Xcc and other microorganisms under natural conditions need to be determined. There is a need to develop more effective, but environmentally compatible, CBCD management measures. This should include development of resistant commercial citrus cultivars, improved disease forecasting systems and effective bactericides and more effective Xcc eradication measures. The potential for biological control of Xcc with naturally-occurring or genetically engineered microbes has not been fully evaluated. Comprehensive citrus health programs need to be established in many tropical countries to generally improve citriculture.

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Characteristics of *Pseudomonas solanacearum* and related bacteria from banana and plantain in South East Asia

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Investigations on two neglected bacterial diseases of banana and plantain in South East Asia are described. In Indonesia, blood disease is a lethal, systemic vascular infection affecting many banana cultivars whereas in the Philippines bugtok disease is a partially-systemic and non-lethal infection of the floral raceme common in cooking (ABB) varieties. Symptoms of both diseases show similarities to those of moko disease in Central and South America (*Pseudomonas solanacearum* race 2); both are caused by non-fluorescent pseudomonads and are probably transmitted by insects. Phenotypic characteristics of isolates from both blood and bugtok diseases were compared with 48 isolates of *P. solanacearum* from moko disease in Central and South America. All bugtok isolates, and most moko isolates, conformed to biovar 1 and were pathogenic both to banana plantlets and to solanaceous hosts. Blood disease isolates showed a distinctive colony morphology and did not readily metabolise glucose and several other carbon sources utilised by biovar 1; all were pathogenic to banana but none produced symptoms in Solanaceae. Numerical analysis of 78 phenotypic characters showed that bugtok isolates clustered with one of two groups of moko isolates, related at 89% similarity, whereas blood disease isolates formed a separate group with 66% similarity to moko strains. These results indicate that the bugtok bacterium is a strain of *P. solanacearum* race 2 but support designation of the blood disease bacterium as a separate taxonomic group.

INTRODUCTION

Until recently, reliable reports of moko disease, caused by *Pseudomonas solanacearum* race 2, were restricted to Central and South America, where it is thought to be a "new encounter" disease of cultivated *Musa* originating in wild *Heliconia* spp. In the late 1960's, moko was reported from newly-developed dessert banana plantations in the southern Philippines, where it is thought to have been introduced in contaminated planting material from Central America (Buddenhagen, 1986). This paper draws attention to some lesser known vascular bacterial diseases that are apparently endemic to South East Asia, and discusses the relationships between the causal bacteria.

BLOOD DISEASE

Blood disease (*penyakit darah*) was first reported by Gäumann (1921, 1923) from the Indonesian island of Sulawesi (formerly Celebes), where it caused the abandonment of newly established dessert banana plantations. The disease was identified in wild *Heliconia* spp. in an undisturbed state and was apparently endemic to the island. Gäumann identified the causal agent as a bacterium which he described as *P. celebensis*, but none of his original isolates survives.

The disease was rediscovered in 1987 (Eden-Green & Sastraatmadja, 1991) when investigations of a previously unknown disease in West Java subsequently led to a survey in Sulawesi which showed that blood disease was still widespread and well known there. Symptoms are very similar to moko, with leaf yellowing, flaccidity and collapse; destruction of fruits, internal vascular discolouration and a reddish coloured bacterial ooze from which the name of the disease presumably originates. Infection is systemic and usually spreads throughout the rhizome affecting the young suckers which may show wilting and act as a source of infection. The disease is particularly common on ABB varieties with dehiscent bracts, such as *pisang kepok* (syn. bluggoe) where there is often evidence of inflorescence infection, presumably spread by insects visiting the male flower bud. It also affects other ABB and AAA varieties including *p. susu, p. nangka, p. ambon, p. raja*, and Gäumann reported finding over 100 local cultivars susceptible to the disease. Blood disease is spreading at rates more than 25 km per year in Java and poses a

serious threat to S E Asia unless appropriate quarantine measures are enforced.

BUGTOK DISEASE

A disease known locally as *bugtok* or *taporuk* was first reported by Roperos (1965) from the S W province of Negros Oriental, where it was said to be widespread in cooking bananas such as saba and cardaba (ABB or BBB types). It was subsequently described in Mindanao in the late 1960's (Zehr & Davide, 1970) and has been the subject of recent studies by the Philippines Bureau of Plant Industry (BPI; Garcia, 1984; Soguilon, 1990). The disease is widespread throughout banana growing regions, in contrast to moko which was first reported in 1969 and remains confined to certain areas of S Mindanao, mainly in dessert bananas.

Unlike blood disease, the symptoms of bugtok are usually confined to the floral raceme and foliar symptoms are rare or absent. Fruits of affected plants are discoloured red or brown, and this is associated with vascular discolouration which does not usually extend far into the lower part of the fruit stem. Hence daughter suckers are rarely affected and the disease is not thought to be spread in planting material. Infection apparently occurs via the inflorescence and there is some evidence that this may occur through both the male and female flowers, in which case the usual practice of removing the male flower bud (denavelling) would fail to control spread of the disease. Recent surveys by the BPI have shown that in some areas bugtok disease causes very high losses of cooking bananas, which are an important staple food, particularly in times of adversity. The disease is not recognised as such in dessert bananas but the causal bacterium is pathogenic to dwarf Cavendish on experimental inoculation.

CHARACTERISTICS OF BLOOD, BUGTOK AND MOKO DISEASE BACTERIA

Cultural and biochemical characteristics of isolates of the blood disease bacterium (BDB) and the bugtok bacterium were compared with those of an extensive collection of strains of moko from the Americas (Table 1). Cultural

TABLE 1

Characterization of banana isolates, UK, 1990

		No. of isolates
From *Musa* and *Heliconia*:		
Americas	(bv1)	43
"	(bv3/4)	5
India	(bv1)	4
Indonesia	(BDB)	13
Philippines	(bv1 - Bugtok)	13
From other hosts:		
groundnut	(bv3)	1
ginger	(bv4)	1
Physalis	(bv3)	1
potato	(bv2)	1
Strelitzia	(bv3)	2
tomato	(bv1)	1

TABLE 2

Percentage of blood disease (BDB), Bugtok and *P. solanacearum* isolates positive; 1990 data, UK

			P. sol	
	BDB	Bugtok	bv1	bv3/4
Nitrate reduction	0	100	98	100
Utilisation of:				
D-galacturonic acid	0	100	100	100
D-gluconate	0	100	100	100
DL-glycerate	0	100	100	100
acetate	0	100	100	100
D-saccharate	0	100	100	90
sucrose	8	100	100	100
myo-inositol	0	100	66	90
L-threonine	0	100	89	100
L tryptophan	0	100	87	90
mucate	0	100	94	100
p-hydroxybenzoate	0	100	60	100
L-citrulline	92	0	66	90
hippurate	100	100	100	10
D(+)galactose	100	0	0	100
trans-aconitate	100	0	0	0

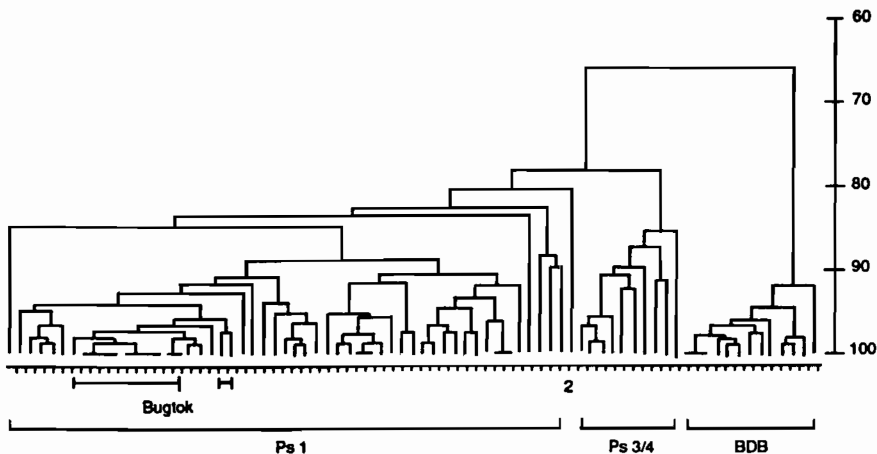

FIGURE 1

Group average linkage cluster analysis of similarity coefficients of *P. solanacearum* (PS) biovars 1,2,3&4, bugtok, and blood disease (BDB) isolates.

TABLE 3

Pathogenicity of isolates from banana and plantain

Origin		Percentage isolates pathogenic to:			
		banana only	banana & tomato/ eggplant	tomato/ eggplant	not patho-genic
Americas	(bv1)	4	74	18	4
Americas	(bv3/4)	0	0	100	0
Philippines	(Bugtok)	0	91	0	9
Indonesia	(BDB)	100	0	0	0

characteristics of bugtok bacteria and moko isolates of *P. solanacearum* were very similar on TZC medium, but colonies of BDB were small and non-fluidal on this and other commonly used media. Isolates of BDB did not utilise glucose or reduce nitrate on first isolation; cells were consistently non-motile and intracellular sudanophilic inclusions could not be demonstrated. A numerical analysis of results of more than 70 phenotypic properties showed that blood disease isolates formed a discrete phenon, whereas bugtok strains could not be distinguished from moko isolates of *P. solanacearum* (Figure 1). Some of the disguishing characteristics of the strains are shown in Table 2.

Results of pathogenicity tests also distinguished BDB from bugtok and moko isolates. Whereas the two latter were pathogenic to both dwarf Cavendish banana plantlets and to seedlings of common solanaceous hosts, BDB did not give symptoms in tomato and eggplant (Table 3).

CONCLUSIONS

These data, supported by recent nucleic acid characterisation studies (S E Seal, unpublished results), indicate that BDB strains are closely related to *P. solanacearum* but have probably evolved independently. Gäumann's original nomenclature, "*P. celebensis*" is no longer valid but these results may warrant the redescription of BDB as a new species. In contrast, bugtok strains could not be differentiated from *P. solanacearum* and probably share a common evolutionary origin with at least one group of moko strains from Central America.

The hypothesis that bugtok in smallholder cooking bananas and moko in dessert banana plantations are, in fact, two diseases in the Philippines caused by the same organism may not be as unreasonable as it might at first appear from consideration of symptoms. The two types of cultivation are quite distinct: large plantations actively discourage the presence of smallholder bananas, owing to the risk of infection with virus diseases such as bunchy top. Thus the likelihood of mechanical cross infection, by cutting tools etc., must be comparatively small. Similarly, the risks of airborne infection through inflorescences must be greatly reduced in commercial plantations by the high degree of efficiency achieved in denavelling operations. Hence the ecology of the two diseases seems to be quite distinct and separate.

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Disease assessment in Madagascar of sheath brown rot of rice caused by *Pseudomonas fuscovaginae*

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ABSTRACT

Sheath brown rot, caused by *Pseudomonas fuscovaginae*, is the main pathogen of highland rice in Madagascar. Conditions of the outbreak and evolution of this disease in the field are partially known, but some epidemiological aspects remain unknown. To study them, disease assessments were made at two sites : Sambaina at 1650 metres altitude and Soanindrariny at 1800 m. The disease incidence (percentage of diseased panicle sheaths per plant) and the disease severity (average percentage of infected tissues per plant) were measured during the heading stage, the period of disease susceptibility. Additionally, to check infection by *Pseudomonas fuscovaginae* in the field, samples were collected and tested in the laboratory. Statistical analysis of these data showed that : at plant level the relation between the square root of the severity and the incidence is linear; at field level the distribution of the disease is uniform; and in geographical distribution the incidence and severity increase with altitude and also in relation to the season (i.e.; time). Differences in disease rates between locations (altitude) and time (progress of the season) could be due to a combination of environmental factors and pathogen variability. This hypothesis will be tested by studying serological and pathogenicity variability in randomly collected isolates.

INTRODUCTION

Pseudomonas fuscovaginae was first reported on rice in northern Japan in 1976 (TANII *et al.*, 1976). Since 1985, this pathogen has been described in Burundi on rice (DUVEILLER *et al.*, 1988), sorghum and maize (DUVEILLER *et al.*, 1989), in Madagascar (ROTT *et al.*, 1989) and Latin America (ZEIGLER and ALVAREZ, 1987) on rice, and in Mexico on wheat (DUVEILLER and MARAITE, 1990). Seed-transmission of *Pseudomonas fuscovaginae* has been described and the treatment of seeds was found to be an effective control method of the disease in Japan (MIYAJIMA, 1988).

In Madagascar sheath brown rot is the main pathogen occurring on irrigated rice at high altitude (ROTT *et al.*, 1991). In this country, among hundreds of cold-tolerant cultivars only a few local cultivars exhibit field tolerance to *Pseudomonas fuscovaginae* (DECHANET *et al.*, 1990). They are used in a breeding programme for producing resistant cultivars. But as base data for this breeding work it was decided to obtain further information on some epidemiological aspects of the disease.

MATERIALS AND METHODS

The population used in the recurrent breeding programme was evaluate in two different locations. The first one at Sambaina (altitude 1650 m) included 5000 plants and second one at Soanindrariny (1800 m) included 4800 plants. Disease assessments were made at four dates during March to April 1992.

At the first date, the SRSD (stratified random sample design) sampling pattern was used (DELP *et al.*, 1986b). The experimental field in Sambaina was divided into 25 equal sectors and the field in Soanindrariny into 24. In each sector 10 plants were observed. At the three other dates the sampling pattern was of the "X" design (DELP *et al.*, 1986b).

Incidence (percentage of diseased panicle sheaths per plant) and severity (average percentage of infected tissues per plant) were assessed on the four dates and at both locations. To calculate the severity, diseased panicle sheaths were recorded according to a visual scale based on estimation of the area of infected tissues (SEEM, 1984).

Within-field distribution of the disease was assessed only on the first date. It was measured by the ratio of within-field variance to mean number of infected plants and with Lloyd's index of mean patchiness (DELP *et al.*, 1986a). For these two measurements a value of 1 indicated a random distribution of the disease; a value greater than 1 indicated an aggregated distribution; and a value smaller than 1 uniform one.

To check presence in the field of *Pseudomonas fuscovaginae*, 30 diseased sheaths were randomly collected on each date. Each sample was separately ground in sterile distilled water (10 mL for 1 g of fresh tissue) and macerated for 30 minutes. Maceration juices were diluted from 10 to 10^9 and 50 µL of each dilution were spread onto two plates of King's B semi-selective medium (ROTT *et al.*, 1989). After 2 and 5 days of growth, colonies were observed. Maceration juices were also tested in DDAS-ELISA (Direct Double Antibody Sandwich, Enzyme Linked Immuno-Sorbent Assay) with purified IGg from the antiserum against the *Pseudomonas fuscovaginae* strain GR2 from Madagascar (ROTT *et al.*, 1989).

RESULTS

Within-field distribution of the disease. The percentage of diseased plants at the beginning of the study was 28% at Sambaina and 52% at Soanindrariny (Table 1). Aggregation measurements indicate uniform distribution of the disease in the field at the two locations. Variance to mean ratio and Lloyd's index of mean patchiness are both less than 1.

Table 1. Aggregation measurements of the sheath brown rot of rice in two locations of Madagascar.

Location (elevation)	Diseased Plants (%)	Variance to mean ratio	Lloyd's index of mean patchiness
Sambaina (1650 m.)	28	0.7	0.9
Soanindrariny (1800 m.)	52	0.3	0.8

Incidence and severity assessment. For each of the four dates and each of the two locations incidence and square root of severity are correlated. These eight regressions are not significantly different at the P=0.1 level. But they are different at the P=0.05 level. Correlation coefficient calculated with all data is 0.75 ; it is highly significant at the P=0.05 level. The value of slope coefficient of linear equation is 0.72+/-0.03 (Fig.1).

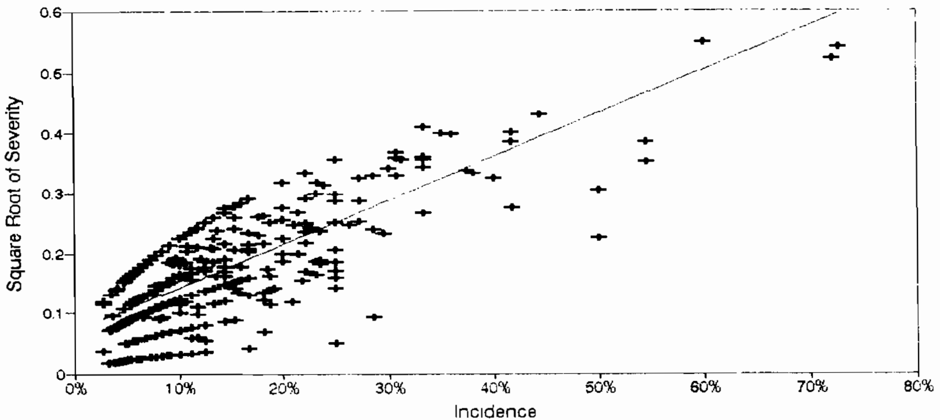

Fig.1. Relations between the square root of severity and incidence. The linear equation is calculated with all recorded data.

In the two locations the average incidence and average severity increased with time (Fig.2). In 33 days, at Sambaina (1650 m), the average incidence increased linearly from 2.4% to 8.8% and average severity from 0.4% to 2.7%. At Soanindrariny (1800 m) the average incidence increased from 6.8% to 18.1% and average severity from 1.5% to 6.8%.

At the latter location, a marked increase occurs between the 14th and the 21st days when 50% of tillers are at the heading stage.

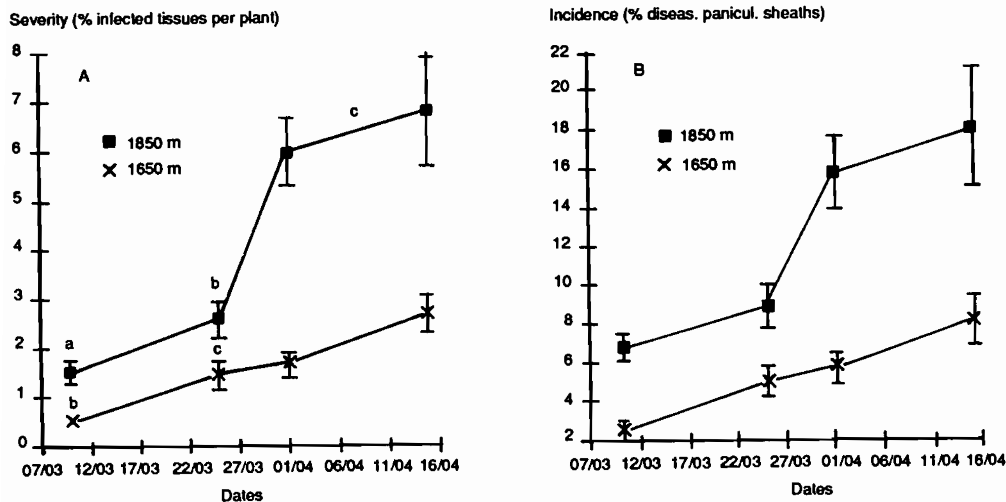

Fig.2. Variation of severity (A) and incidence (B) with dates at Soanindrariny (1800 m) and at Sambaina (1650 m). Each point is the mean value and the bar is the standard error of the mean. Letter a represents 10% of tillers at the heading stage; b, 50%; c, 100%.

Presence of *Pseudomonas fuscovaginae*. Among the 240 diseased samples tested, 62% from Soanindrariny and only 53% from Sambaina had a positive reaction in ELISA. From each sample, one, two, or three different types of colonies were isolated on KBS medium. For samples exhibiting both a positive reaction in ELISA and only one type of colony, the features of these colonies were always the same ; they are fluorescent under ultra-violet light, up to 2-3 mm in size, white in colour, and convex. They formed a crystal in bacterial culture. This colony type was isolated from all samples exhibiting a positive ELISA reaction. This type of colony was also isolated from 100% of samples exhibiting a negative ELISA reaction collected at Soanindrariny and from 36% of those collected at Sambaina.

DISCUSSION

At plant level, the severity of the disease is correlated with its incidence irrespective of altitude or time. Because disease is assessed on plants differing genetically from one another, the incidence-severity relation seems to depend only on the genetics of the plant-pathogen relation (SEEM, 1984). If incidence represents the pathogen penetration in plant and severity its progression in tissues, the incidence-severity relation seems to indicate a relation between pathogen ability to penetrate and its ability to progress in tissues. This hypothesis is confirmed

by previous experiments carried out under controlled conditions on cultivars inoculated with different *Pseudomonas fuscovaginae* strains. In these experiments classification of strains based on the number of diseased plants or on the average necrosis size are identical (Jaunet, *unpublished data*).

At field level the disease distribution is uniform. This distribution can be due to the high percentage of diseased plants (DELP *et al.*, 1986a). Seed-transmission of *Pseudomonas fuscovaginae* has been reported in Japan where seed treatments are effective (MIYAJIMA, 1988). Seed treatment experiments carried out at Soanindrariny and Sambaina reveal no significant differences between treated and untreated seeds (Jaunet, *unpublished data*). Moreover, seeds of recurrent breeding population come from a low-altitude region where *Pseudomonas fuscovaginae* is not reported. Thus, disease outbreak is not due to a seed transmission. The origin of primary inoculum remains unknown at Madagascar.

Disease development appeared to differ with altitude. Average incidence and average severity are greater at 1800 m altitude than at 1650 m. This difference could be attributed to differences in climate or in *Pseudomonas fuscovaginae* population pathogenicity. All samples collected at Soanindrariny and 89% of those collected at Sambaina gave colony types corresponding to *Pseudomonas fuscovaginae* (ROTT *et al.*, 1989). But with serological differences. Serological variability of *Pseudomonas fuscovaginae* with antiserum for GR2 strain had been previously reported (ROTT *et al.*, 1991). Pathogenicity of isolated strains and its relation with serological reaction will be studied in the near future. Average incidence and average severity rise more slowly at Sambaina than at Soanindrariny. Indeed, at this latter location notable increase occurs when more than 50% of tillers are at the heading stage, while no increase appears at the same stage at Sambaina. So, these findings confirm on the one hand that rice is more susceptible to *Pseudomonas fuscovaginae* during the heading stage and, on other hand, that temperature is an important factor of this susceptibility (MIYAJIMA, 1983). However the effects of temperature remained partially unknown. Previous experiments carried out under controlled revealed a direct effect of temperature on the pathogenicity of *Pseudomonas fuscovaginae* strain inoculated on a cultivar, that increases with day temperature decrease (Jaunet, *unpublished data*). The world distribution of *Pseudomonas fuscovaginae* relates the occurrence of the pathogen to low temperatures. The disease increase with altitude in Madagascar provides further evidence of this relation.

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Pathogenic bacteria associated with rice seed samples from Indonesia and Nepal

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ABSTRACT

Pathogenic bacteria were commonly isolated from rice seeds collected from rice growing areas of Indonesia and Nepal. They were detected by direct plating of seeds in agar medium, growing-on tests and liquid assays. All bacteria were seed transmitted and readily caused symptoms in seedlings within 4–10 days. The sheath brown rot pathogen, *Pseudomonas fuscovaginae* was isolated by seedling tests, liquid assays and direct platings. Another fluorescent pseudomonad was also detected in Nepalese seeds and identified as *P. marginalis* (*P. fluorescens* biotype II). Both bacteria were highly pathogenic when inoculated into several rice cultivars. This is the first record of *P. fuscovaginae* from rice seeds from Indonesia and of *P. marginalis* from Nepal. *P. avenae*, *P. glumae* as well as *X. campestris* pvs. *oryzae* and *oryzicola* were detected from seedling tests and liquid assays. Additionally a non-fluorescent *Pseudomonas* sp. and *Erwinia* sp. were detected from Indonesia and Nepal, respectively. Several pathogenic fungi were present in the infected seed samples including the sheath rot pathogen *Sarocladium oryzae*. Mixed infections of 2 to 4 bacteria were recorded. *P. glumae* and pathogenic xanthomonads were seldom found as single infections in the seed.

***Keywords*: seed-borne pathogenic bacteria, testing methods, seed transmission, mixed infections.**

INTRODUCTION

Rice seeds are extensively exchanged among countries, regions and continents along with seed-borne pathogens including bacteria. Different pathogenic bacteria have been isolated from rice seeds (Goto *et al.*, 1988; Agarwal *et al.*, 1989).

Pseudomonas avenae, P. glumae, Xanthomonas campestris pvs. *oryzae* and *oryzicola* are important bacterial pathogens of the rice crop in Indonesia and Nepal. Besides, *Erwinia chrysanthemi* pv. *chrysanthemi* and *E. herbicola* have been found in Indonesia while *Erwinia* sp. (Manandhar, 1987) and *P. fuscovaginae* (H.K. Manandhar, C. Nieves Mortensen & B.J. Thapa, 1991, accepted for publication by IRRN) have been reported from Nepal.

A new disease, bacterial red stripe (BRS) of rice has been described from Indonesia (Mogi, 1988). The causal agent has been preliminary identified as a *Pseudomonas* sp. Losses of 16-76% of rice grain has been reported. The disease present in different areas of Indonesia (Sumatra, Java, Bali, Lambok and South Kalimantan) has also been reported from Vietnam (Du *et al.*, 1991). Disease symptoms include circular or oval-shape lesions of green yellowish or brown yellowish colour. Lesions of 3-5mm diameter enlarge gradually and brown-orange or red stripes rapidly progresses to the upper part of the leaf blades. The disease develops rapidly at tillering stage and infected leaves wither before maturity.

Bacterial diseases of rice are of economic importance in Indonesia and Nepal, but there are no official regulations in both countries for testing rice seeds for seed-borne pathogenic bacteria. The present studies were initiated to determine the seed health status of rice seeds from Indonesia and Nepal. Our need for testing rice seeds led us to evaluate different methods that could enable the detection of not only single infections but also mixed bacterial infections. Other studies herein included deal with the evaluation of susceptibility of Nepalese cultivars to fluorescent pseudomonads, and growing-on tests with *Pseudomonas* infected seeds. Bacterial cultures (obtained from Indonesia) isolated from plants showing BRS symtoms were characterized.

MATERIALS AND METHODS

Rice seed samples. Seventy-two samples of 21 varieties collected from different parts of Indonesia (West, East and Central Java, Bali, North Sumatra, South East Sulawasi, South Kalimantan, North Sumatra and Jakarta) during 1987-88 and 1989-90 were used in the studies. Twenty-seven Nepalese seed samples of 23 varieties collected from research farms and farmers' fields during 1989-90 were included.

Reference isolates. Isolates of *Erwinia stewartii* (SS 104), *Pseudomonas avenae* (Nepal), *P. fuscovaginae* (BCE 32 and BCE 3), *P. glumae* (NCPPB 2391), *P. plantarii* (AZ 84236, 8523, 8371, 8202 and 03.01723) *Xanthomonas campestris* pv. *oryzae* (NCPPB 1936 and 1150), and *X.c.* pv. *oryzicola* (NCPPB 1585, 1632, 1151 and B 911) were used as references.

Antisera. Crude polyclonal antisera against *P. avenae* Nepal, *P. fuscovaginae* BCE-32, *P. glumae* NCPPB 2391, *X.c.* pv. *oryzae* 1536, and *X.c.* pv. *oryzicola* B 912 were used.

Detection methods

Seedling symptoms test. One hundred to two hundred seeds per sample were grown in slide cassette holders (Shakya and Chung, 1983). Seedlings showing disease symptoms were recorded within 14 days. Loopfuls of bacterial suspensions from macerated diseased tissue were streaked onto KB agar medium plates.

Liquid assays. Four-hundred ground-up rice seeds per sample were suspended in 200 ml 0.85% sterile saline and incubated for 2, 4 and 20 h at room temperature with periodical shaking. Samples were serially diluted and aliquots of 0.05 ml were plated onto KB,

SPG (Thushima *et al.*, 1986) and GF medium (Nieves Mortensen, 1989) and incubated at 25-27°C for 3-5 days.

Direct plating of seeds. Two to four hundred rice seeds per sample (washed in running tap water for 1 h) were partly embedded in KB agar plates (25 seeds/Petri dish of 9 cm diameter) and incubated at 25-27°C for 24-48 hours.

Identification. The purified isolated bacteria were first subjected to preliminary tests such as gram reaction (KOH solubility), fluorescent pigment on KB, and yellow pigment on YDC. Isolates were tested for pathogenicity followed by physiological and serological tests. Bacterial cells were observed in the electron microscope.

Inoculation tests. Inoculations were made on 21-60 days-old rice plants of cultivars Oryzica 1, IR 64, and Taichung Native 1 grown from heat treated seeds (65°C for 6 days). Bacteria (10^{8-9} cfu/ml) were inoculated by stem injections or by the clipping method. Inoculated plants were placed in polythene bags and incubated at 27-30°C with 12 h day light. Symptoms were recorded after 3, 7 and 21 days. Tobacco, pepper and tomato leaves were infiltrated with bacterial suspensions.

Physiological tests. Pathogenic isolates were tested for oxidase reaction, arginine dihydrolase, levan production, gelatin liquefaction, oxidative/fermentative metabolism, nitrate reduction, lipase activity, H_2S production, and utilization of carbohydrates (Lelliott & Stead, 1987). Presumptive diagnosis of fluorescent bacteria was performed by the LOPAT tests.

Serological tests. Most of the isolates were tested by slide agglutination (antisera dilution 1:10 in saline) and Ouchterlony double diffusion tests.

Growing-on tests. Two seed samples, IR 64 (Acc. No. 28470) and Semeru (Acc. No. 31474) collected from fields showing BRS symptoms were grown in individual pots at 28-30°C with 12 h light. Both samples were initially infected with *P. fuscovaginae*. Isolations and observations were made from plants at different growth stages.

Varietal reactions of rice to fluorescent pseudomonads. Fifteen rice varieties/lines from Nepal (Atte, Bhangeri Marshi, Bhuin Dhan, Chainung Sen Yu, Junge, Khumal-2, Khumal-4, Masuli, Raksali, Himali, Chhomro, Taichung 176, NR 1463, NR 10157, and NR 10067) were evaluated for their resistance against Nepalese isolates of *P. fuscovaginae* and *P. marginalis*. Thirty-days-old plants were inoculated by stem injections.

Seed health testing for fungi. Twenty-five seeds (400 seeds per sample) were placed in Petri dishes of 9 cm with 3 layers of wet blotter paper and incubated at 21°C ±1 under NUV light for 7 days. Seeds were examined and recorded individually under the binocular microscope for the presence of fungi.

RESULTS

Seedling symptom tests. Phytopathogenic bacteria were consistently isolated from seedlings grown in slide cassette holders (Table 1). Mostly *P. avenae* and *P. fuscovaginae*

were found in single infections. *P. avenae* was commonly found in association with *P. fuscovaginae, P. glumae, X. c.* pvs. *oryzae* and *oryzicola*. Mixed infections of *P. avenae* and *P. glumae* and *X.c.* pv. *oryzae*, or *X.c.* pv. *oryzicola* were also detected. In one seed sample *P. avenae, P. fuscovaginae, P. glumae* and *X.c.* pv. *oryzae* were recorded together.

Table 1. Percentage of bacterial infections of rice seeds and infected rice seed samples from Indonesia tested by seedling symptom tests

Pathogen	% infected seedlings[a]		% infected seed samples[a]	
	1987–1988	1989–1990	1987–1988	1989–1990
Pseudomonas avenae	91.0	52.0	1.5–48.0	1.0–12.0
P. fuscovaginae	21.0	29.0	0.5–48.0	1.0–10.0
P. glumae	16.0	14.0	0.5–6.5	1.0–12.0
Pseudomonas sp.	4.0	ND	0–9.5	ND
Xanthomonas campestris pv. *oryzae*	38.0	8.0	0.5–34.0	1.0–4.0
X.campestris pv. *oryzicola*	8.0	6.0	1.5–12.5	1.0–3.0

a. 1988–1989: 24 seed samples; 1989–1990: 48 seed samples. ND- Not detected

Liquid assays. Isolations from ground-up seed extracts revealed *P. avenae, P. glumae, X.c.* pvs. *oryzae* and *oryzicola*. Fluorescent pseudomonads (*P. fuscovaginae* and *P. marginalis*) were also detected in Nepalese samples. Similar recovery values were obtained after 2 and 4 h of incubation. After 20 h bacteria could not be detected. *P. avenae* and *P. glumae* ($2x10^3$ - $2x10^6$ cfu/ml) were readily isolated from the infected samples (42 and 33%, respectively). Recovery values of $2x10^5$–$4x10^5$ cfu/ml were recorded for *X.c.* pvs. *oryzae* and *oryzicola*. Mixed infections of *P. avenae* and *P. glumae* type A and B (Tsushima *et al.*, 1986) could be detected from platings on SPG medium. *X.c.* pv. *oryzae* was often isolated from Nepalese seed samples (26%) collected from plain areas with sub-tropical climate including one sample (var. Himali) from Kathmandu (mid hills)(Table 2).

Direct plating of seeds. Only *P. fuscovaginae* was detected from the Indonesian seed samples (Semeru, IR 42, IR 64, and IR 70) collected during 1989–1990 . The infection ranged from 1-4%. *Erwinia* sp., *P. fuscovaginae, P. marginalis* (*P. fluorescens* biovar II), and *X.c.* pv. *oryzae* were detected from the Nepalese samples. Fluorescent pseudomonads were common in the Nepalese samples (30%) collected from high hills (Lumle and Pakhribas).

Growing-on tests. Seedlings raised from two of the seed samples showed initial brown discoloration and stripes on coleoptiles, leaves and sheaths. Yellow discoloured seedlings were also observed which usually died within 21–32 days. Brown stripes were observed on the leaf blade of IR 64 mature plants raised from seed with 10% of infection with *P. fuscovaginae*. Dry necrotic lesions surrounded by chlorotic areas were observed at flowering stage. Such lesions expanded throughout the leaf blades which became dry within 4 days.

In the second seed sample with 2% of infection of *P. fuscovaginae*, 9 and 11% of the grown plants showed brown sheath rot symptoms at booting and flowering stage, respectively.

Table 2. Detection of phytopathogenic bacteria from 27 Nepalese rice seed samples

Bacteria	Method	% of infected seed samples	Origin[1]
Erwinia sp.	Direct plating of seeds	7.4	Eastern high hills
Pseudomonas avenae	Liquid assays	14.8	Mid hills and plains
P. glumae	Liquid assays	11.1	Mid hills
P. fuscovaginae	Liquid assays	3.7	Eastern high hills
	Direct plating of seeds	11.1	Eastern high hills
P. marginalis	Liquid assays	7.4	Western mid and high hills
	Direct plating of seeds	29.9	Western mid and high hills
Xanthomonas cam-	Direct plating of seeds	3.7	Plain areas
pestris pv. *oryzae*	Liquid assays	25.9	Plain areas[2]
X.c. pv. *oryzicola*	Liquid assays	3.7	Plain areas

1. Areas from where those seeds were collected, high hills - cool temperate, mid hills - warm temperate, plains - sub-tropical climate. 2. plus 1 sample from mid hills (Kathmandu).

Infected plants did not produce panicles or produced panicles with empty and/or discoloured grains. Symptomless plants survived and produced panicles with discoloured grains. *P. avenae* and *P. fuscovaginae* were isolated from the brown stripe of coleoptiles and brown discoloured sheaths. Harvested discoloured grains showed infections with *P. fuscovaginae* and *Sarocladium oryzae*.

Characterization of bacterial isolates. Characteristics of isolates from BRS infected leaves obtained from Indonesia, and a group of isolates from Nepalese seeds are presented in Tables 3 and 4, respectively. The Indonesian isolates were found to be close to *Erwinia herbicola* while the Nepalese were identified as *Pseudomonas marginalis* (*P. fluorescens* biovar II).

Reactions of rice varieties/lines to fluorescent pseudomonads. All tested varieties/lines showed susceptibility to *P. fuscovaginae* isolated from Nepalese seeds. Only line NR 10157 was moderately resistant. The variety Bhuin Dhan including NR 1463 and NR 10157 lines were found moderately resistant to *P. marginalis* (isolated from Nepalese seeds) while the others were susceptible. Both tested bacteria were more virulent compared to the reference isolate BCE 32 of *P. fuscovaginae*. Seventeen per cent of the plants died 10-15 days after inoculation. Several plants with retarded growth produced secondary tillers after 15-21 days of inoculation.

Pathogenic fungi. *Alternaria padwickii, Bipolaris oryzae, Cercospora oryzae, Fusarium moniliforme, Microdochium oryzae, Pyricularia oryzae* and *Sarocladium oryzae* were commonly detected in both Indonesian and Nepalese seed samples. *A. padwickii* was

detected in more than 92% of the samples.

Table 3. Characteristics of bacterial isolates from BRS infected leaves obtained from Indonesia

Character	Indonesian isolates	*Erwinia herbicola* pv. *herbicola**
Gram reaction	–	–
Flagella	peritrichous	peritrichous
Growth on MS medium	+	ND
Pink pigment on YDC	–	–
Oxidase	–	–(D)
Anaerobic growth	+	+
Sorbitol utilization	+	+(D)
Arginine dihydrolase	+	ND
α-methyl-glucoside	+	–
Nitrate reduction	+	+(D), v(S)
H_2S from cysteine	+	+
Indole production	–	–(D)
Lipase	–	ND
Gelatin liquefaction	v	+
Starch hydrolysis	–	+(D)
Potato soft rot	–	–
Tobacco hypersensitivity	–	ND
Hypersensitivity in pepper and tomato plants	+	ND

* Data taken from Schroth & Hildebrand (1988)(S), Dye (1983)(D), + = positive reaction, – = negative reaction, v = variable reaction among isolates, ND = no data available.

Table 4. Characteristics of bacterial isolates identified as *Pseudomonas marginalis* from Nepal

Character	Nepalese isolates	CIAT isolates*	*Pseudomonas marginalis**	*P. fuscovaginae**
Gram reaction	–	–	–	–
Flagellation	>1, polar	>1, polar	>1, polar	>1, polar
Yellow pigment	–	–	–	–
Fluorescent pigment	+	+	+	+
Arginine dihydrolase	+	+	+	+
Gelatin liquefaction	+	+	+	+
Oxidase	+	+	+	+
Levan production	+	+	+	–
Starch hydrolysis	–	–	v	(+)
Lipase	+	+	+	+
Nitrate reduction	+	v	+	–
Potato soft rot	(+)	+	+	–
Tobacco hypersensitivity	+	+	ND	ND
Growth at 4°C	(+)	–	+	–

* Data taken from Zeigler & Alvarez (1987), + = positive reaction, (+) = weakly positive, – = negative reaction, v = variable reaction among isolates, ND = no data available.

DISCUSSION

Different pathogenic bacteria even in mixed infections could readily be detected from the seeds by seedling symptom tests with the advantages of determining the seed-transmitted ones. However, the method is considered unsuitable when numerous seed samples are to be tested since it is time consuming and laborious.

P. avenae was the most commonly isolated bacterium not only in single infections but also in associations with *P. fuscovaginae, P. glumae, X.c.* pvs. *oryzae* and *oryzicola. P. fuscovaginae* was detected for the first time in Indonesian rice seeds (IR 64) of 1987-1988 harvest. It was the only pathogen detected by direct plating from Indonesian seeds, however, other bacteria could be detected by this method from Nepalese seeds.

Liquid assays favoured isolations from seeds with single infections. Isolations were difficulted when samples harboured large number of saprophytic bacteria and when the selectivity of the plating medium was low.

The characterization of the bacteria isolated from plants with BRS symptoms showed that they belong to the Enterobacteriaceae, probably *Erwinia herbicola* which was also confirmed by the Identification and Taxonomic Services, IMI, Surrey, England. *E. herbicola* has been commonly isolated from rice plants and earlier considered a saprophyte. Our isolates were able to induce symptoms of yellow and brown discoloration (after 21 days) in inoculated plants and hypersensitive reactions in tomato and pepper leaves but not in tobacco leaves. In any case the symptoms induced were different from the ones described for the BRS disease. We assume that the isolates were not identical to those described by Mogi (1988) from BRS infected plants.

The BRS pathogen could not be isolated from those mature plants which showed necrotic lesions surrounded by chlorotic areas (growing-on tests). Instead *P. fuscovaginae* was isolated from such infected plants and seeds while the fungus *Sarocladium oryzae* could be detected from discoloured grains. These pathogens may play an important role in the "dirty panicle" disease (Zeigler & Alvarez, 1987).

Our studies reveal that many seed-borne pathogenic bacteria of rice are also seed-transmitted along with fungal pathogens. This also suggests the confusion in diagnoses based on symptoms alone or in testing methods that do not favour the isolation of a given pathogen. Recent outbreaks of bacterial diseases and spread of relatively new pathogens reflects the evident need of testing for seed-borne phytopathogenic bacteria. The value of testing will also be emphasized when control measures to reduce further spread of the pathogens should be undertaken.

The presence of *P. fuscovaginae* in seeds from Indonesia and Nepal and susceptibility of Nepalese rice cultivars to the bacteria indicates the potentiality of spread of fluorescent pseudomonads in those rice growing areas.

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Bacterial sheath brown rot of rice caused by *Pseudomonas fuscovaginae* in Nepal

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ABSTRACT

Symptoms very similar to bacterial sheath brown rot of rice caused by *Pseudomonas fuscovaginae* were noted in many paddy fields of hilly regions between 1300 to 2300 meters. The percentage of affected tillers with sheath brown rot symptom was 25 to 80. A fluorescent pseudomonad was consistently isolated from affected sheaths, grains and seedlings raised from these seeds. Based on pathogenicity, biochemical and serological tests, the bacterium was identified as *Pseudomonas fuscovaginae* (TANII *et al.*, 1976 ; MIYAJIMA *et al.*, 1983). This study reports for the first time *P. fuscovaginae* causing bacterial sheath brown rot of rice in Nepal.

INTRODUCTION

Nepal is known to cultivate rice at highest altitude in the world. Of the total rice cultivated land, 21% lies in mid-hills (1300 to 1600 m) and high hills (1650 to 2650 m). Bacterial sheath brown rot of rice caused by *Pseudomonas fuscovaginae* is known to be widespread at high altitudes, in northern Japan (TANII *et al.*, 1976 ; MIYAJIMA, 1983) in high lands of Burundi and Madagascar (DUVEILLER *et al.*, 1988 ; ROTT and NOTTEGHEM, 1989) as well as in Latin American countries (ZIEGLER and ALVAREZ, 1987). The pathogen causes sheath rot, necrotic lesions on leaf sheath and sterility of spikelets. This disease has been reported to be a serious and widespread when cool humid conditions prevails during booting stage (MIYAJIMA, 1983). Symptoms very similar to bacterial sheath brown rot of rice described by various authors (MIYAJIMA, 1983 ; ZIEGLER and ALVAREZ, 1987) had been noted in paddy fields of hilly regions in Nepal during the senior author's field visits since 1985. The cause of such symptoms had been considered mainly due to fungus *Sarocladium oryzae* or cold climatic conditions (MANANDHAR, 1987). However, due to consistent isolation of fluorescent pseudomonads from rice plants affected with such symptoms, it was suspected that the symptoms might be of bacterial cause rather than fungal and an investigation was undertaken.

This study reports for the first time *P. fuscovaginae* causing bacterial sheath brown rot of rice in Nepal (preliminary report submitted to IRAT = Institut de Recherches Agronomiques Tropicales et des Cultures Vivrières in 1990). This investigation was a collaborative project with IRAT/CIRAD (CIRAD = Centre de Coopération Internationale en Recherche Agronomique pour le Développement) supported by the European Economic Community Research Contract TS2-0046-B (GDE).

MATERIALS AND METHODS

Collection of plant materials

Farmers paddy fields at high altitude (ranging from 1300 to 2300 m) were visited at booting stage in the month of August and before harvest in the month of October to November. Plant materials and seeds were collected from the suspected sheath brown rot infected plants.

Isolation of bacteria

Pieces of diseased sheath and rice seeds were plated on King's B semi-selective (KBS) medium (ROTT *et al.*, 1989) and moist blotter. Plates were incubated at 26°C. After 1 to 3 days of incubation, bacterial growth around the materials producing fluorescent pigment were purified on KB medium. Some seedlings (4-7 days old) grown on moist blotters developed water soaked dark brown lesions or blotches on leaf sheaths and leaves. Pieces of necrotic seedling sheath as well as infected sheaths from the field were macerated in sterile water and streaked on (KBS) medium. Purified fluorescent isolates were stored in sterile water at 4°C for routine work.

Identification

Pathogenicity test was performed on 3-4 weak old rice seedlings of cultivar Taichung by injecting bacterial suspension approximately 10^8 cells/ml. Biochemical tests were carried by conventional procedures (SCHAAD, 1988). Serological test using Enzyme-Linked Immunosorbent Assay (ELISA) with *P. fuscovaginae* monoclonal antibodies (mabs) was applied to confirm the identification (BENEDICT *et al.*, 1989).

RESULTS AND DISCUSSION

Symptoms and distribution

Incidence of bacterial sheath brown rot like disease were noted in most of the paddy fields at high altitudes. Rice plants at booting stage were found affected with dark or reddish brown lesions on flag leaf sheaths, sheaths and panicles. Lesions on sheath were usually found extending its full length. Symptoms on flag leaves were diffuse resulting in a brown to maroon blotching. The diseased panicles which were with sterile and discoloured or rusty grains had been usually found enclosed by severely affected flag leaves. The percentage of affected tillers with brown rot symptom was 25 to 80 based on random observation taken per hill (10 hills per field plot in each locality where disease incidence was noted). With much discussion, several investigators had reported these types of symptoms mainly caused by the bacterium *P. fuscovaginae* (TANII *et al.*, 1976 ; MIYAJIMA, 1983 ; ROTT and NOTTEGHEM, 1989 ; ZIGLER and ALVAREZ, 1987).

Isolation and identification

Fluorescent bacteria were consistently isolated from leaf sheaths, grains and seedlings raised from seeds. The isolation of bacteria from seeds and other parts of plant materials treated with 70% ethyl alcohol was very successful. Fungal growth was not found on sheath pieces plated on moist blotter or KBS. On KB medium the suspected colonies of *P. fuscovaginae*, were creamy white, circular, raised and butyrous with entire margins while some colonies were flat and translucent. Most of these isolates were pathogenic to rice seedlings and positive on tobacco hypersensitivity test except a few isolates of the flat type colonies. Isolations had been made from several cultivars collected from different localities. Pathogenic bacterium was readily reisolated.

The biochemical characteristics of these pathogenic isolates were very much similar to the characters of *P. fuscovaginae* given by ROTT and NOTTEGHEM, 1989 (Table 1) and also agreed with diagnostic characters described by MIYAJIMA *et al.*, 1983., except for utilization of sorbitol and potato soft rot being negative in their description. In contrast with *P. marginalis* (ROTT and NOTTEGHEM, 1989), present isolates were positive on tobacco hypersensitivity and negative on levan production, use of polygalacturonic acid and Beta-Glucosidase. Thus on these basic biochemical and pathogenicity tests, the strains isolated from rice plant materials from paddy fields of Nepal were identified as *P. fuscovaginae* (TANII *et al.*, 1976 ; MIYAJIMA *et al.*, 1983).

Table 1

Comparison of the characters of pathogenic fluorescent isolates of rice bacterial sheath brown rot from Nepal with published characters of *Pseudomonas fuscovaginae*

Character	Isolates (Nepal)	*P. fuscovaginae*[a]
Gram reaction	-	-
Fluorescent pigment	+	+
Levan production	-	-
Kovac's oxidase	+	+
Potato soft rot	+	-
Arginine dihydrolase	+	+
Tobacco hypersensitivity	+	+
Beta-glucosidase	-	ND
2-Ketogluconate	V	-
Polygalacturonid acid	-	-
Utilization of (Growth on)		
D-Arabinose	-	-
L-Arabinose	+	+
Ayers	-	ND
Belanite	+	ND
Glucose	+	+
Inositol	-	-
Mannitol	+	+
L-Rhamnose	-	-
Saccharate	+	ND
Sorbitol	+	-
Trehalose	+	+
Cellobiose	-	-
Fructose	+	+

[a] ROTT and NOTTEGHEM, 1989

+ = Positive , - = Negative , V = Variable reaction , ND = Not done

Table 2

P. fuscovaginae strains isolated from rice plant materials collected from different localities in Nepal

Number of strains	Cultivar	Locality	Altitude	Collected year
12	Himali	Dolkha	2300 m	1987
2	Pokhareli	Dolkha	2000 m	1987
2	Local	Dolkha	2000 m	1990
3	Local	Sankhu	1350 m	1990
3	Taichung	Panga	1450 m	1991
2	Masuli	Tokha	1400 m	1991
1	Taichung	Kirtipur	1350 m	1991

Eight strains reacted positively only with *P. fuscovaginae* 195-14-1-1 monoclonal antibodies (mabs), while the other two mabs did not react with any of the strains. The identification of other pathogenic strains had been confirmed by slide agglutination tests with *P. fuscovaginae* 6801 antiserum.

Isolation of *P. fuscovaginae* from rice cultivars collected from different localities at hilly regions (Table 2) clearly indicated that the sheath brown rot of rice in Nepal is of bacterial cause rather than fungal and the disease is widespread.

The present investigation affirms for the first time an incidence of bacterial sheath brown rot disease of rice caused by *P. fuscovaginae* in Nepal. This disease seems to be one of the major constrain of rice cultivation at high altitude of the country. The disease had also been noted in some parts of the low lands. Further investigations on over-all survey and yield losses are urgently needed.

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Bacterial blight of rice in Nepal

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ABSTRACT

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (hereafter Xoo) is the major disease of rice in Nepal. The disease was first recorded from Balaju in 1968. Since then it has occurred both in the Terai and Hills. The current commercial cultivars Masuli, Bindeshwori, and CH-45, all introduced in Nepal are highly susceptible to BB. Severe epidemics developed in 1979 and 1989. Grain yield was reduced by 10-30% in 1989 and attributed to an increase in the proportion of unfilled grains and reduction in 1000-grain weight. The relationship between bacteriological and pathological characteristics were studied. No distinct cluster among 53 Xoo strains was detected by numerical analysis. All strains were associated at 82.40% similarity coefficient in the dendrogram. Pathogenic specialization of Xoo was observed in Nepal. The strains were highly virulent to IRRI differentials, as evidenced by the high proportion of susceptibility of CAS209 (*Xa-10*) and IR1545-339-2-2 (*xa-5*). Rice cultivar, Laxmi, was resistant to many strains. No correlation was observed among these characters. Additional testing of Nepal Xoo strains with DNA probes is in progress.

Additional keywords : disease severity, disease resistance, virulence, differential interaction, epidemiology, numerical analysis.

INTRODUCTION

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* is one of the most destructive diseases of rice in Nepal (ADHIKARI & SHRESTHA, **1989**). The disease has been reported as early as **1968** in Kathmandu Valley and is now widely prevalent throughout the Terai belts (plain) and Hills.

Considerable variation has been observed among the strains of *X. o.* pv. *oryzae* in other rice growing countries in relation to bacteriological characteristics

(HARTINI *et al.*, 1976; SHEKHAWAT & SRIVASTAVA, 1968; VERA CRUZ *et al.*, 1984), virulence (GUPTA *et al.*, 1986; MEW, 1987; MEW & VERA CRUZ, 1979; NODA *et al.*, 1990), monoclonal antibodies (BENEDICT *et al.*, 1989), and restriction fragment length polymorphisms (LEACH & WHITE, 1991; LEACH *et al.*, 1990). A set of differential cultivars with specific genes for resistance has been used as the basis for race designations (MEW & VERA CRUZ, 1979). As a result, six races of *X. o.* pv. *oryzae* have been described in the Philippines (MEW, 1987). In Nepal, research on bacterial blight is in its infancy. Information on bacteriological and pathological characteristics are therefore unknown.

MATERIALS AND METHODS

Bacterial strains. The strains collected from various areas in Nepal are listed in Fig. 1. The cultures were maintained at -4 C and transferred regularly during the course of the experiments.

Biochemical and physiological tests. The 53 strains used in this study and inocula were prepared by suspending 10 ml sterile distilled water and standardized to 2.1×10^8 colony forming units (cfu)/ml. The methods used for determining the biochemical and physiological characters of the cultures have been described by several authors (DYE, 1962; HAYWARD, 1964; HUGH & LEIFSON, 1953; KOVAC, 1956; SIERRA, 1957). For numerical analysis, all tests were scored as positive (coded 2) or negative (coded 1). Both positive and negative matches were determined in the calculation by unweighted average pair-group method (SNEATH & SOKAL, 1973).

Virulence tests. Thirty-five strains were evaluated on eight rice cultivars to measure variation in virulence. These were the five IRRI differentials, IR8 (possessing *Xa-11* resistance gene), IR20 (*Xa-4*), IR1545-339-2-2 (*xa-5*) (henceforth referred to as IR1545), DV85 (*xa-5, Xa-7*), and CAS209 (*Xa-10*). Three Nepalese cultivars, Himali, Laxmi, and Sabitri, were selected on the basis of preliminary studies which indicated differential interactions with the bacteria. Inoculum was prepared from 2-day-old cultures by suspending bacterial mass in 10 ml sterile distilled water and adjusted at 2.8×10^8 cfu/ml. Three fully expanded young leaves were clip-inoculated with each bacterial strain at 35 days after sowing. Lesion development from the inoculated leaf tip of each cultivar

was recorded in centimeter (cm) at 21 days after inoculation. Data on lesion length of each cultivar-strain combination was analyzed and comparisons of main and interaction effects between the rice cultivar and the bacterial strain were calculated.

RESULTS

Clustering of strains. All strains were associated at 82.40% S_{sm} (Fig. 1). Thirteen features were positive to all 53 strains investigated; that is, all strains were aerobic, catalase positive; grew at 10, 20, and 30 C; hydrolyzed starch, egg yolk, and Tween 80; produced levan; liquefied gelatin; reduced and peptonized litmus milk, and produced acid from glucose. The following tests were negative to the 53 strains: Gram stain, acetoin production, growth at 5 and 40 C; growth in anaerobic conditions; nitrate reduction; oxidase activity; growth in the presence of 0.01% (w/v) and 0.05% (w/v) tetrazolium chloride salt; hydrolysis of Tween 20; oxidase activity; indole production; alkalinization of litmus milk; acid production from maltose and lactose, growth in the presence of tetracycline (30 mcg), penicillin (10 μ), cloxacillin (5 mcg), and 0.05% (w/v) crystal violet. Several tests, such as growth on glucose concentrations, basic fuchsin; 0.1% (w/v) tetrazolium salt; crystal violet; acid production from carbohydrate and sensitivity to sisomycin (10 mcg); chloramphenical (30 mcg); co-trimoxazole (25 mcg), ampicillin (10 mcg), and erythromycin (15 mcg), varied with the strains.

Virulence tests. Lesion lengths ranged from 2.07-19.66 cm on IR8, 4.80-19.08 cm on IR20, 3.25-19.33 cm on IR1545, 0.08-22.25 cm on CAS209, 0.27-15.99 cm on DV85, 0.05-20.24 cm on Himali, 0.05-14.75 cm on Laxmi, and 0.26-14.66 cm on Sabitri. Rice cultivars also differed significantly in their degree of resistance to *X. o.* pv. *oryzae*. Using the five IRRI differentials and three Nepalese rice cultivars, the 35 strains were classified into nine pathogenic races. Significant ($P < 0.01$) cultivar-strain interaction on lesion length was demonstrated. Race 1 consisted of 19 strains which were highly virulent to all rice cultivars (Table 1).

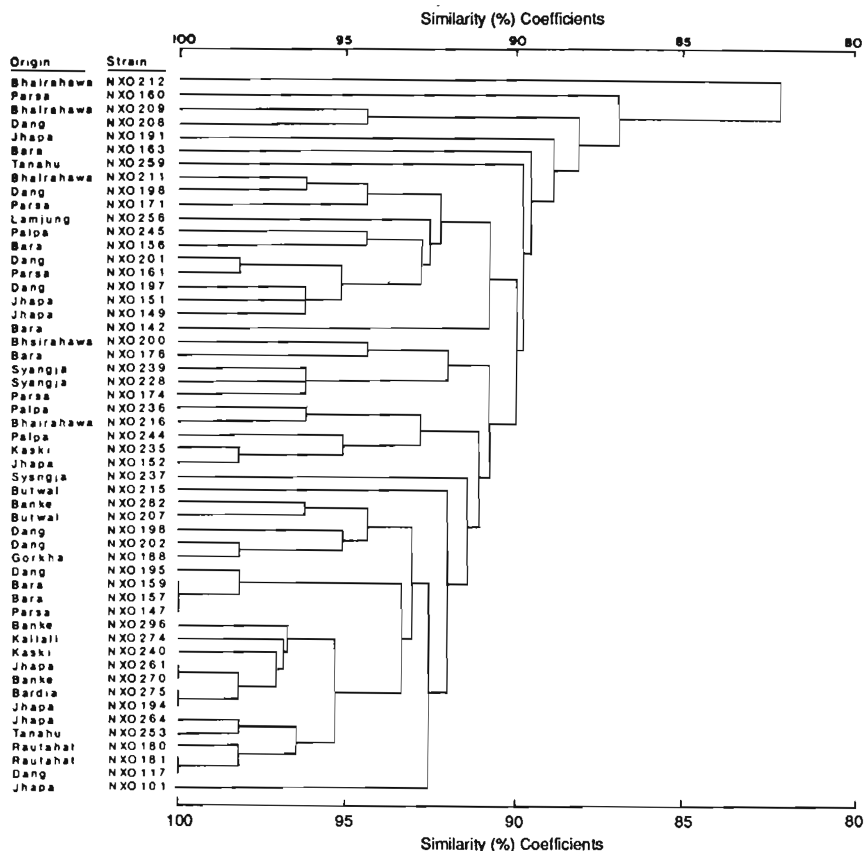

Fig. 1. Dendrogram of similarities coefficients clustered by unweighted average pair-group method showing phenotypic similarities among 53 strains of X. o. pv. oryzae collected in Nepal.

DISCUSSION

The numerical analysis of phenotypic features indicated no striking differences in biochemical and physiological traits among the strains, confirming the existence of homogeneous populations. The results were similar to the previous studies (TSUCHIYA *et al.*, 1982; VERA CRUZ *et al.*, 1984). Variation in terms of acid production from trehalose and sucrose, growth on 0.005% tetrazolium chloride and 0.05% (v/w) crystal violet and sensitivity towards antibiotics were observed in this study. Such phenotypic variation among the strains of the present investigation should be compared with strains of *X. o.* pv. *oryzae* from other countries using a few determinative tests described herein

Table 1. Virulence of representative strains of *X. o.* pv. *oryzae* to rice cultivars in Nepal.

Strain	Race[b]	Lesion length (cm) on rice cultivar[a]							
		IR8 (Xa-11)	IR20 (Xa-4)	IR1545 (xa-5)	CAS209 (Xa-10)	DV85 (xa-5, Xa-7)	Himali (Unknown)	Laxmi (Unknown)	Sabitri (Unknown)
NXO201	1	10.70 bcd	11.77 bc	13.15 abc	10.67 c-g	12.52 abc	9.12 abc	11.70 a	6.50 ab
NXO211	1	19.08 a	14.00 ab	17.33 a	17.50 ab	5.00 def	11.83 ab	7.5 a-d	8.77 a
NXO149	2	9.87 ab	10.02 bcd	11.30 abcd	12.80 b-g	9.19 b-e	8.92 a-d	6.83 a-e	0.29 b
NXO174	2	9.33 bcd	10.55 bcd	9.45 be	8.26 d-g	10.80 bcd	3.33 cde	5.52 a-f	2.45 ab
NXO156	3	11.08 bcd	12.83 bc	11.79 abc	10.71 b-g	10.49 bcd	12.10 a	0.37 ef	1.60 b
NXO209	3	8.65 bcd	8.85 bcd	4.13 de	7.69 efg	11.00 bcd	8.41 a-d	1.89 def	0.26 b
NXO198	4	12.37 b	12.60 bc	10.35 a-e	14.50 bcd	0.69 f	10.63 ab	5.11 a-f	7.12 ab
NXO210	4	12.03 bc	6.42 cd	3.25 e	6.87 fg	0.27 f	7.16 a-d	6.63 a-f	4.80 ab
NXO151	5	8.33 bcd	19.16 a	15.00 ab	13.00 b-f	14.50 ab	2.61 de	10.00 ab	4.44 ab
NXO159	6	10.71 bcd	10.25 bcd	3.43 de	10.04 d-g	1.33 f	10.06 a	2.23 c-f	3.64 ab
NXO195	6	12.83 b	11.35 bcd	10.74 a-e	11.37 b-g	2.50 f	10.96 ab	2.87 c-f	4.91 ab
NXO181	7	11.41 bc	10.60 bcd	12.87 abc	14.12 b-e	1.54 f	11.80 ab	0.05 f	0.76 b
NXO199	8	3.36 de	11.22 bcd	3.65 de	0.08 h	14.37 ab	0.05 e	3.50 b-f	1.55 a
NXO205	9	2.07 e	14.61 ab	6.99 cde	13.83 b-e	0.37 f	0.56 e	0.27 ef	2.35 ab

[a] Lesion length was measured at 21 days after inoculation. Lesion length <3 cm = R and >3 cm = susceptible.
[b] Race 1 consisted of 19 strains; race 2, five strains; race 3, three strains, race 4, two strains; race 5, one strain; race 6, two strains and race 7, 8 and 9, composed of only one strain each. In column, means followed by a common letter are not significantly different according to Duncan's multiple range test ($P < 0.05$).

together with those of earlier studies (SHEKHAWAT & SRIVASTAVA, 1968; VERA CRUZ & MEW, 1989).

The results demonstrated significant cultivar-strain interaction, suggesting pathogenic specialization of *X. o.* pv. *oryzae* in Nepal. Using three IRRI differentials, IR8, CAS209, and DV85, and Nepalese rice cultivars, Himali, Laxmi, and Sabitri, the 35 strains were classified into nine pathogenic races. The strains differed widely in their virulence and should be considered to represent pathogenic races. Race 1 commonly isolated in this study was similar to the Philippine race 6 strains of *X. o.* pv. *oryzae* (MEW, 1987). The pathogenic specialization of *X. o.* pv. *oryzae* has also been reported from other rice growing countries on the basis of specificity on rice differential cultivars (GUPTA *et al.*; MEW, 1987; MEW & VERA CRUZ, 1979; NODA *et al.*, 1990). Comparisons of restriction fragment length polymorphisms of genomic DNA of the pathogen populations from broad geographic areas are in progress. With adequate monitoring of the race dynamics of the pathogen populations and a better understanding of the epidemiology of the pathogen, successful control strategies based on selective deployment of resistance genes could be developed for pathogen control (LEACH & WHITE, 1991).

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A disease of garlic caused by a fluorescent pseudomonad closely related to *Pseudomonas fuscovaginae*

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Abstract. A disease of garlic consisting of leaf and sheath necrosis sometimes leading to soft rot and plant death is associated with several fluorescent pseudomonads, many of them classified as *Pseudomonas fluorescens* biotypes 1 and 5. Artificial inoculation experiments showed that only bacteria belonging to the second group (*P. fluorescens* biotype 5) could be regarded as pathogenic on garlic. When compared to three strains of *Pseudomonas fuscovaginae* (two from rice, one from wheat), almost 80 biochemical properties were similar. Rice sheaths inoculated with strains from garlic exhibited symptoms identical to those caused by the three *P. fuscovaginae* strains and the latter induced necrosis when infiltrated into garlic leaves. Garlic strains gave no positive reaction when tested with a *P. fuscovaginae* antiserum (immunodiffusion and immunofluorescence). Considering that serological properties may be variable in *P. fuscovaginae*, it is suggested that the pathogenic strains isolated from garlic are closely related to *P. fuscovaginae*.

Keywords : *Pseudomonas fluorescens* biotype1- *Pseudomonas fluorescens* biotype 5- *Pseudomonas fuscovaginae*- Garlic- *Allium sativum*-"Café au lait" disease- Reunion Island.

In Reunion Island, garlic (*Allium sativum*) is sometimes affected by a disease the symptoms of which are similar to those of the "café au lait" disease, caused by *Pseudomonas fluorescens* biotype 1 in France (SAMSON, 1982) and in Italy (CALZOLARI & BAZZI, 1985): leaf and sheath necrosis sometimes leading to soft rot and, in severe cases, death of the plant. In the last years, several types of fluorescent pseudomonads were isolated from diseased garlics in Reunion. Those which could induce a hypersensitive reaction when infiltrated into tobacco leaves were retained for further studies (GIRARD, 1990). Most of them were initially identified as *Pseudomonas fluorescens* biotype 1 and *P. fluorescens* biotype 5 when studied according to different methods (FAHY & LLOYD, 1983; PALLERONI, 1984). In the present study, several strains of both biotypes were

compared to reference strains of phytopathogenic bacteria with which they seemed to be related according to biochemical properties.

Materials and methods. Twenty-five strains of *P. fluorescens* biotype 1 and nine strains of *P. fluorescens* biotype 5 isolated from diseased garlic plants in Reunion were studied. They were compared to one strain of *P. fluorescens* biotype 1, causal agent of "Café au lait" disease (CFBP[1] 2022) and three strains of *P. fuscovaginae* (CFBP 2065 and 3084, isolated from rice in Japan and from wheat in Mexico respectively, and GR2, isolated from rice in Madagascar, supplied by ROTT (CIRAD-IRAT, BP 5035, 34032 Montpellier-Cédex, France).

Microbiological characteristics. For each strain the following tests were performed : tobacco hypersensitivity; levan production; arginine dihydrolase; Kovac's oxidase; gram staining; catalase; nitrate reduction; denitrification; aerobic growth; esculin, cellulose, gelatin, starch, tween 80 hydrolysis; acid production from erythritol, sorbitol, sucrose, D+trehalose, mannitol, dulcitol, adonitol, inositol; urease, indol, acetoin production (GARDAN & LUISETTI, 1982); pit formation on polypectate gel (HILDEBRAND, 1971); 2 keto-gluconate production (LELLIOT & STEAD, 1987) ; sensitivity to colistin was determined with Pasteur discs (50µg) on Mueller-Hinton medium (BARRY, 1980). Moreover, the utilization of carbohydrates was studied on API 50 galleries for the biotype 5 strains and the *P. fuscovaginae* strains as previously described (GARDAN *et al.*, 1984).

Pathogenicity tests. Two types of inoculation techniques were performed on garlic. The first one consisted of infiltrating 0.5 ml of a bacterial suspension in sterile distilled water (sdw) adjusted to 10^8 cfu/ml into the leaves of three garlic plants per strain. The second method consisted of pouring 0.5 ml of a bacterial suspension in 0.5% tween20 adjusted to 5.10^8 cfu/ml into the leaf whirl of garlic plants at the 5-6 leaf stage (20 plants per strain). In this case, symptoms were regularly rated using a 0-5 scale: 0, no symptom; 1, chlorosis or lesion on one leaf; 2, one dried leaf; 3, two dried leaves; 4, three dried leaves or more; 5, plant dead (SAMSON, personal communication).

Two tests were performed using the second technique, the first one under natural conditions in a "shade house" in summer season (average temperature 26+/-6°C, spray irrigation three times a day) with five biotype 1 and four biotype 5 strains, the second one in a climatic chamber (20+/-0,5°C, 90-95% RH, 12 hour photoperiod) to study the pathogenicity of the *P. fuscovaginae* strains on garlic.

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[1] Collection Française de Bactéries Phytopathogènes, Station de Pathologie Végétale et de Phytobactériologie, INRA, BP 57, 49071, Beaucouzé Cédex, France.

The pathogenicity tests on rice consisted of injecting a bacterial suspension prepared in sdw (10^8 cfu/ml) into 20 three-week-old rice seedlings (ROTT *et al.*, 1991). Rice seedlings were then kept under controlled conditions (13+/-0.5°C, 90-95%RH, 12 hour photoperiod).

Serology . Biotype 1 strains were tested against a polyclonal antiserum for one strain of "Café au lait" disease supplied by SAMSON (INRA, BP 57, 49071, Beaucouzé Cédex, France). Biotype 5 strains were tested against a polyclonal antiserum for strain GR2 of *P. fuscovaginae* supplied by ROTT. Two serological techniques were used: Ouchterlony's double diffusion on slides and immunofluorescence (SCHAAD *et al.*, 1990).

Results. *Biotype 1 strains*. The 25 local biotype 1 strains differed from the reference strain CFBP 2022 by the following characteristics (Table 1): gelatin liquefaction, tween 80 hydrolysis, acid production from adonitol, tobacco hypersensitivity, pathogenicity on garlic and serology. All the other characteristics were identical.

Most of the strains gave positive results when infiltrated into garlic leaves, but there was a range of variations in the severity of the symptoms: only 4 local strains showed symptoms identical to those of the reference strain CFBP 2022 (complete necrosis of the infiltrated leaf after 48 hours); 7 strains only induce a yellowing of the infiltrated area and 14 strains gave an intermediate reaction (wilting of the infiltrated area). The local strains tested by pouring a bacterial suspension into the leaf whirl induced the death of the oldest leaves and, in some cases, a temporary distortion and discoloration of the youngest ones.

Biotype 5 strains. Results were variable for the following tests only: gelatin liquefaction; tween 80 hydrolysis . Only strain GR2 reacted with the antiserum for strain GR2 (Table 2). These characteristics are considered to be variable from one strain to another for *P. fuscovaginae* (DUVEILLER *et al.*, 1988; ROTT *et al.*, 1991). All the local strains tested and the *P. fuscovaginae* reference strains induced the complete wilting of the leaves when infiltrated at 10^8 cfu/ml after 24-48 hours. Two local biotype 5 strains and the *P. fuscovaginae* reference strains induced the drying of all leaves and sometimes the death of the plants when poured into the leaf whirl of garlic plants. They were also pathogenic on rice seedlings. The other local strains tested induced the drying of the oldest leaves and a temporary distortion of the youngest ones. The disease severity for the most pathogenic strains on garlic and rice is schematized (Fig. 1 and 2).

Table 1 Comparison between biotype 1 local strains and "café au lait" strain CFBP 2022.

	Strain 2022	25 local strains
Gelatin liquefaction	-	+
Tween 80 hydrolysis	-	+
Acid production from adonitol	+	-
Tobacco HR	+	v
Garlic leaf infiltration	+	v
Bacterial suspension in leaf whirl	+	+/- (*)
Serology	+	-

(+), positive; -, negative; +/-, doubtful positive; v, variable; (*), 5 strains tested only .

Table 2: Comparison between biotype 5 local strains and *P. fuscovaginae* strains.

Positive tests : Aerobic growth ;arginine dihydrolase; catalase; fluorescence on King B; Kovac's oxidase; oxidative metabolism of glucose; acid production on mannitol; growth on glycerol, L arabinose, ribose, D xylose, galactose, D glucose, D fructose, D manose, mannitol, trehalose, D arabitol, gluconate; resistance to colistine; tobacco HR.

Negative tests: Levan production; nitrate reduction; denitrification; esculin, cellulose and starch hydrolysis; acid production from erythritol,sorbitol, sucrose, dulcitol, adonitol, inositol; urease, indole, acetoin and 2 keto-gluconate production; growth on erythritol, D arabinose, L xylose, adonitol, ß methyl-xyloside, L sorbose, rhamnose,dulcitol, inositol, sorbitol, alpha-methyl-D glucoside, N acetyl-glucosamide, amygdaline, arbutine, esculine, salicine, cellobiose, maltose, lactose, mellibiose, saccharose, inuline, melizitose, D rhamnose, starch,glycogene, xylitol, gentiobiose, D turanose, D lyxose, D tagatose, D fucose, L fucose, D arabitol, 2 keto-gluconate, 5 keto-gluconate; pit formation on polypectate gel pH 5, 7 and 8.5.

Variable tests: Gelatine liquefaction; tween 80 hydrolysis; serology.

Discussion and conclusions. Further investigation is needed to conclude whether the biotype 1 local strains isolated from garlic are actually involved in the disease: their pathogenicity on garlic has not been undoubtfully proved yet, although some of them can induce symptoms when infiltrated into garlic leaves. They differ from the "café au lait" reference strain 2022 by several characteristics However, it is surprising to notice that all the local strains are resistant to colistin,

like the pathogenic strains of "café au lait "disease (SAMSON,1982) whereas most of the biotype 1 non pathogenic strains tested by SAMSON appeared as susceptible to that antibiotic.

On the other hand, several biotype 5 local strains are pathogenic to garlic and rice. They are closely related - if not identical - to *P. fuscovaginae* : most of the microbiological features -including inability to produce 2 keto-gluconate - are the same and the symptoms both on garlic and on rice cannot be distinguished from those induced by the 3 *P. fuscovaginae* reference strains. None of the local strains reacted when tested against the antiserum prepared with GR2 reference strain, but neither did the two other reference strains 2065 and 3084. To our knowledge, this is the first time that a fluorescent pseudomonad closely related to *P. fuscovaginae* is reported as pathogenic to garlic.

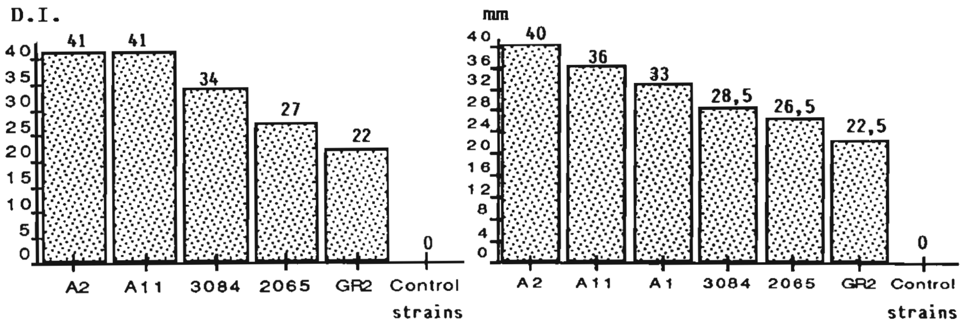

Figure 1 Figure 2

Figure 1. Disease index (D.I.) of garlic 7 days after inoculation with 2 strains of *P. fluorescens* biotype 5 (A2; A11) and 3 strains of *P. fuscovaginae* (CFBP 2065; CFBP 3084; GR2)

D.I.=$\Sigma$is-Σit

$\Sigma$is: Sum of the marks of each plant inoculated with strain 's'

Σit : " " " " " " " " " 0.5% tween 20 only

20 plants per strain; each plant evaluated according to a 0-5 scale.

Figure 2. Average length in mm of the necrosis induced on rice seedlings 6 days after inoculation with 3 strains of *P. fluorescens* biotype 5 (A2; A11; A1) and 3 strains of *P. fuscovaginae* (CFBP 2065; CFBP 3084; GR2)

20 plants per strain; control plants inoculated with a non pathogenic *P. fluorescens* biotype 5 strain.

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Genetic variation in Indian isolates of *Xanthomonas campestris* pv. *oryzae*

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Abstract

Using 7 differentials, TKM6 (xa4), IR1545 (xa5), DV85 (xa7), CAS209 (xa10), IR8 (xa11), BJ1 (xa13) and Tn1 (xa14) 17 Indian isolates of *Xanthomonas campestris* pv. *oryzae* were tested for pathogenicity. There were nine bacterial groups based on pathogenicity in the above cultivars. Using genomic clone library prepared from another Indian isolate, DNA:DNA hybridization experiment was carried out. Though it revealed variation in the hybridization pattern there was no correlation between pathogenicity variation and variation in hybridization pattern.

Key Words: *Xanthomonas campestris pv.oryzae*, variation, DNA:DNA hybridization.

Xanthomonas campestris pv.oryzae is the causal agent of bacterial blight (BLB) disease of rice. BLB occurs globally from Asia to Africa and the Americas. Its distribution ranges from 20 S in Queensland in Australia, to 58 N in Heilang Jiango, China and from sea level to the Tibetan Plateau. Usually it is more prevalent in the wet season than in dry season, and in low land than in favorable upland conditions. In breeding bacterial blight resistance, as in other diseases, two independent variables: the rice cultivar and the pathogen play crucial role. Until 1980 there are more than 3,000 rice cultivars identified to be resistant to a common strain of the bacterium in IRRI (MEW, 1987). Whether these rice cultivars are resistant to all strains or to only a certain strains, in the country is not known. A study was made on selected Indian isolates of *X.c.*pv.*oryzae* for variation.

Materials and Methods

Bacterial cultures and culture conditions

Isolates of *X.c.*pv.*oryzae* (XCO) were made from infected rice plants and characterized according to BRADBURY (1986). Strains were maintained in Wakimato's potato agar medium (WAKIMATO, 1967). For prolonged storage, strains were frozen in 15 % glycerol at -70 C (MANIATIS et al, 1982). Luria broth was used to grow the bacterium for testing pathogenicity.

Isolation of genomic DNA of *X.c.*pv.*oryzae*

The culture was grown in 100 ml of LB medium for 12 h. The culture was chilled in ice and the cells were pelleted by centrifugation at 12,000 x g at 4 C for 10 min. Cells were washed twice in ice cold TE buffer and suspended in 40 mL of the buffer. Protease and SDS were added to get a final concentration of 500 µg/mL and 1% (W/V) respectively and the mixture was incubated for 45 min at 0 C before being allowed to warm to 20 C with occasional gentle swirling. The lysate was extracted thrice with TE saturated phenol containing 0.1% 8-hydroxy quinoline. The DNA was precipitated with isopropanol, washed with 70% ethanol and dried under vacuum. The precipitate was dissolved in 10 mL TE, 0.5 mL of 0.1 M spermine was added and the precipitated DNA was collected by centrifugation. Spermine was extracted by suspending the pellet in 10 mL 0.1 M sodium acetate, 3 mM $MgCl_2$ in 70% ethanol. After 1 h, the DNA was centrifuged and the extraction, repeated with 20 ml solution. The DNA was washed with 70% ethanol and dried. The dried pellet was suspended in 1 mL distilled water.

Preparation of pUC18 vector

Plasmid pUC18 was prepared in a maxi scale from 1 liter of overnight culture in LB containing 25 µg/ml tetracycline. Collected cells were suspended in 20 ml of TGE buffer containing 10 mg/ml lysozyme and incubated in ice for 10 min. Forty ml of NaOH-SDS solution was added and incubated in ice for further 10 min. To this 30 ml of 3 M sodium acetate (pH 7.0) was added and the mixture was chilled in a freezer for 15 min. It was centrifuged at 20,000 x g for 15 min. The clear supernatent was added with an equal volume of phenol: chloroform (1:1), mixed well, centrifuged at 5000 x g for 10 min to separate phases. The upper aqueous layer was transferred to a clean tube and extracted with an equal volume of chloroform. To the aqueous phase, 0.1 volume of 3 M sodium acetate (pH 4.8) and 2 volumes of ice cold ethanol were added and incubated in ice for 10 min. The precipitate formed was collected by centrifugation at 8000 x g for 10 min. The pellet was washed with 70 % ethanol, dried under vacuum and dissolved in 1 ml of distilled water. The sample was subjected to CsCl-EtBr density gradient banding. The ccc form of the vector was collected, ethidium bromide was removed by extraction with isoamyl alcohol. The DNA was dialyzed against TE buffer and precipitated with ethanol.

EcoRI digestion of vector DNA

First a 2 μg of the DNA was checked for digestibility. The reaction mixture consisted of : 10 μl pUC18 DNA (2 μg), 1.5 μl of 10 X buffer, 3 μl water and 0.5 μl (4 units) EcoRI enzyme. This was incubated at 37 C for 2 h in a water bath and was run in an agarose gel of 0.7 %. In the adjacent lanes uncut pUC18 DNA and λ DNA were run. Using this reaction conditions the DNA was digested in a maxi scale. For the maxi digestion the reaction mixture consisted of: 100 μl (30 μg) pUC18 DNA, 12.5 μl 10 X buffer, 5 μl water and 7.5 μl of EcoRI (60 units).

The mixture was incubated at 37 C in a water bath for 2 h. The reaction was terminated by placing the tube at 70 C for 10 min. From the digest 2 μl was run in an agarose gel and the cutting was ascertained.

Ligation and transformation

The suitability of the pUC18 vector DNA for cloning was checked for its self ligatability before and after CIP treatment. The reaction mixture consisted of : 25 μl of pUC18 DNA (3 μg), 3 μl 10 X buffer, 1 μl ATP and 1 μl (1 unit) T4 DNA ligase.

After mixing the constituents (before adding the enzyme), 10% of the mixture was kept separately to check the ligated and unligated DNA. The mixture was incubated at 16 C overnight. The ligated sample was run along with the unligated DNA in 0.7% gel.

The EcoRI digested genomic DNA had to be cloned into the vector pUC18. The reaction mixture for the ligation reaction consisted of: 15 μl (1.5-3 μg) of vector DNA, 6.5 μl (1.5 μg) of insert DNA, 1 μl ATP, 2.6 μl 10 X buffer and 1 μl (1 unit) T4 DNA ligase. Before adding the enzyme, 2 μl of the mixture was taken out and kept separately in a microfuge tube. After adding the enzyme, the mixture was incubated at 16 C, overnight. Ten percent of the ligated sample was run along with the aliquot taken before ligation so that the ligated DNA can be compared with non ligated DNA.

Southern transfer and DNA:DNA hybridization

Genomic DNA from the isolates of *X.c.*pv.*oryzae* was isolated (TURNER et al, 1984). Ten μg of the DNA was digested with EcoRI enzyme. The digests were electrophoresed in 1% agarose gel. The resolved fragments were transferred to nitrocellulose filter (MANIATIS et al, 1982).

Labeling and Hybridization

Inserts from the genomic clones were isolated and 2 µg of DNA was labeled with ^{32}P using the Nick translation kit obtained from Promega, Leiden. The labeled inserts were hybridized one by one with the nitrocellulose filter according to MANIATIS *et al* (1982).

Pathogenicity testing was carried out as per standard procedures (ICAR, 1972).

Results and Discussion

When using 7 variables containing the following resistance genes: xa4, xa5, xa7, xa10, xa11, xa12, xa13 and xa14 there is considerable variation in the pathogenicity of the Indian isolates (Tables - 1).

DNA:DNA hybridization experiment was carried out using the genomic clone inserts of *X.c.*pv.*oryzae* as probes. These probes were hybridized to the EcoRI digested genomic DNA (of Indian isolates of *X.c.*pv.*oryzae*) in nitrocellulose filter. This experiment also revealed the presence of considerable variation among the isolates of *X.c.*pv.*oryzae* (Fig.1). A comparative study of five differential varieties from Japan and four from IRRI all carrying specific genes for resistance, was

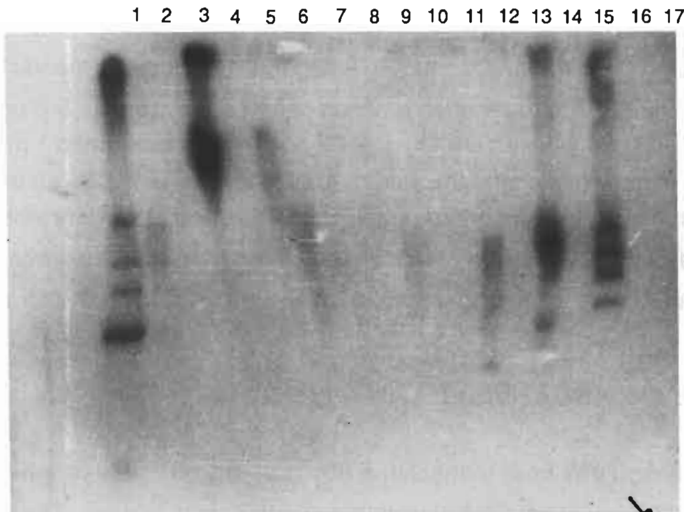

Fig. 1. Southern hybridization of genomic clones with the genomic DNA of *X. c.* pv. *oryzae* isolates. Numbers 1 to 17 represent the isolates XCO1 to XCO17.

Table - 1 Pathogenicity variation of the Indian isolates of *X.c.*pv.*oryzae*

Isolate	TKM6 (xa4)	IR1545 (xa5)	DV85 (xa7)	CAS209 (xa10)	IR8 (xa11)	BJ1 (xa13)	Tn1 (xa14)
XCO1	S	S	S	S	R	S	S
XCO2	S	R	R	S	R	R	S
XCO3	S	S	R	S	R	R	S
XCO4	S	S	S	S	S	R	S
XCO5	S	R	R	S	R	R	S
XCO6	S	S	R	S	S	R	S
XCO7	S	S	S	S	S	R	S
XCO8	R	R	R	R	S	R	S
XCO9	R	R	R	S	R	R	S
XCO10	S	S	R	S	S	S	S
XCO11	R	R	R	R	R	S	R
XCO12	S	S	R	S	S	S	S
XCO13	S	S	R	S	S	S	S
XCO14	R	R	R	R	R	R	S
XCO15	R	R	R	R	R	R	S
XCO16	S	S	R	S	S	S	S
XCO17	R	R	R	R	R	R	S

Analysis of the results showed that there were atleast 9 groups among the isolates tested:

Group	Resistant in cultivar having
1	xa13
2	xa7
3	xa7 and xa13
4	xa7, xa11 and xa13
5	xa7, xa10, xa11 and xa13
6	xa5, xa7, xa11 and xa13
7	xa4, xa5, xa7, xa10 and xa13
8	xa4, xa5, xa7, xa11 and xa13
9	xa4, xa5, xa7, xa10, xa11 and xa13

conducted in Japan and Philippines against pathogenic groups I-V in Japan. The nine bacterial groups were distinct in specific virulence, while each of the differential varieties had a gene or genes different from others in specific resistance (HORINO et al (1981). In Thailand 3 groups were identified on IR8, IR20, IR1545, DV85, RD7 and RD9 (EAMCHIT and MEW, 1982). Using Japanese and IRRI differentials HORINO et al (1981) identified 9 bacterial groups. Our results also indicate the presence of 9 bacterial groups among the Indian isolates of *X.c.*pv.*oryzae*. Though the variation found by the DNA:DNA hybridization study did not correlate with the variation in pathogenicity, it does show that there is considerable variation in the DNA sequences present in the isolates.

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Bacteriosis of onion in the state of Tamaulipass and San Luis Potosi, Mexico

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ABSTRACT

In the municipality González. state of Tamaulipasa, a bacterial soft rot of onion bulbs was detected in 1988-1989 fall-winter season with a total loss of 25 ha. In the 1990-1991 season in the state of San Luis Potosi, incidence of the same disease fluctuated between 5 and 15%. From the internal bulb rot of white, yellow and red varieties, mainly pathogenic bacteria were isolated, characterized and identified as *Erwinia carotovora* subsp. *atroseptica* with some variation of other type of *Erwinia*. This is the first report of *E. c.* subsp. *atroseptica* in Mexico on onion.

KEYWORDS

Erwinia carotovora subsp. *atroseptica*, onion.

INTRODUCTION

Southern region of T amaulipas grows annually between three and six thousand hectares of onion for export. About half way through the growing period (1988-1989, automn-winter), a bacterial disease of onion reaching an incidence of 100% as total or partial internal rot was observed on an extension of 25 ha in the municipality González. The loss amounted to 108.000 dollars.

In the following period (1989-1990) the same problem was observed in the municipalities of Altamira and Mante in the state of Tamaulipas and also in Ebano, state of San Luis Potosi, with an incidence between 5 and 10%.

In 1990-1991 period, the onion bulbs showed again an internal rot in Altamira with an incidence of 5-15% and in Ebano with 11%. The affected commercial lots were grown with the varieties Red Granex (res), Henry Special and Ringer (yellow) and an unknown white variety.

Onion soft rot caused by *Erwinia carotovora* (RODRIGUEZ, 1972) was reported in México in 1972 in the state of Morelos. Since then, onion soft rot in the state of México was reported to be caused by various bacteria such as *Erwinia chrisantemi*, *Pseudomonas cepacia* and *Corynebacterium michiganense* (MANRIQUE and FUCIKOVSKY, 1991). In California, TEVIOTDALE (1989)

reported *P. cepacia* as a cause of onion rot. In Africa, HATTINGH (1981) reported *E. herbicola* causing necrosis of the aerial part of the onion. GITAITIS *et al.* (1991) found *P. viridiflava* causing severe rot of onions in Georgia, U.S.A.

Due to the heavy losses and the unknown cause of this disease, a study was undertaken to identify the pathogen. On the basis of this and other data, there is a possibility to suggest future strategies of control.

MATERIAL AND METHODS

In both periods 1989-1990 and 1990-1991 samples of diseased onions were taken from the fields in the municipalities of González and Altamira, Tamaulipas and Ebano, San Luis Potosi. Fifty onion bulbs were processed in the laboratory in order to note the damage and isolate the bacteria. King's B medium, (SCHAAD, 1988), was used to purify and select the bacteria and later to demonstrate their pathogenicity in triplicate on bulbs maintained at 28°C. Bulbs were punctured with a needle and an inoculum produced on solid medium was inserted. White, yellow and red onions were used for this work which was repeated with potato slices. Ten virulent isolates that produced rot were then characterized using SCHAAD's manual (SCHAAD, 1988). Hypersensitivity was tested on tobacco with $1x10^7$ cells/ml.

RESULTS AND DISCUSSION

Distribution of the diseased bulbs in the field appeared randomly. The symptoms of light soft rot started in the apex of the bulb, spread progessively in the central part along a scale and finally reached the basis of the bulb where it produced a brown colour of the tissue. A soft consistence was noted in many bulbs although many were somewht firm on the exterior.

From all the diseased bulbs only bacteria were isolated and purified. These isolates tested on onion bulbs produced typical symptoms : later, these were reisolated and characterized. Inoculated bulbs and potato slices showed rot usually after 24 hrs, although some variation in the rapidity of the rot was noted between isolates.

Five different isolates did not produce hypersensitivity in tobacco, were Gram negative, motile, without endospore, oxidation-fermentation positive, produced acid from alpha-methyl glucoside, lactose, maltose, palatinose and trehalose, were non fluorescent, liquefied gelatin and produced reticulated colonies under oblique light. Other isolates which showed some variation are in the process of further characterization.

On the basis of these tests, five isolates were identified as *Erwinia carotovora* subsp. *atroseptica* and the other isolates appear to belong to other species of *Erwinia*. This is the first report of onion soft rot caused by *Erwinia carotovora* subsp. *atroseptica* in México. This disease had been previously attributed to *Erwinia carotovora* (RODRIGUEZ, 1972).

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A new bacterial disease of rice and barley from Ranchi (India) due to *Pseudomonas marginalis* (Brown) Stevens

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Abstract

Rice and barley are grown in alternate succession in some areas of Ranchi. On the leaves of rice cvs. IR-36, Rajendra 202 and Mussorie and barley cvs. Karan 4, BAU-2205 and BR-32 initially very small margin spots appeared which enlarged and coalesced length wise to become light to dark brown and brown black lesions. In rice, inflorescence and grains too were covered by them. Water soaking persisted for a while in their leaves. Severe bleaching from tip downwards and chlorosis preceeded spot appearance in barely leaves. In rice it was to the contrary.

In 45d PDI rose from 1.4-2.0% to 27.75 - 32.0% in rice cvs., and in 30d from 2.25-3.75% to 27.75-32.0% in barley cvs. The disease increase rates r and r_1, show cultivar - and duration -specific trends in rice but not in barley. Bacterial numbers recovered from leaves of rice and barley after 40 d and 25 d of infection ranged between 1.92-2.12x10 ^{3} and 1.68-2.02x10 ^{3} CFU/ml respectively.

LOPAT test indicated that rice and barley isolates had the features of a flourescent *Pseudomonas* species. On the basis of acid production from maltose, sucrose and specially purple- lactose and several other features the pathogen has been tentatively identified as *Pseudomonas marginalis* (Brown) Stevens

Key Words

Rice cvs., barley cvs., PDI, r, r_1, spots, lesions, chlorosis, bleaching, LOPAT, *Pseudomonas marginalis*

Methods

The values of PDI, r and r_1 were determined as per formulae laid down earlier (VAN DER PLANCK,1963). Usual diagnostic tests (FAHY & HAYWARD, 1983) for identification of the bacterium were carried out.

Results and discussion

We present here the details of symptoms taken at suitable intervals (table 1) and of disease progress (table 2) in the leaves of rice and barley, and the characteristic features of the bacterial pathogen that cuased this new disease.

Overall trends of symptom expression were similar in both the cereals. However, early stage water soaking in leaves, and late spread out of lesions on inflorescence and grains were discernible in rice only. Severe bleaching from tip downwards and chlorosis preceeded spot appearance in barley; and the reverse was true in rice.

A less known rice disease with brown discoloration of sheath and panicles due to *P. oryzicola* has been recorded earlier (GOTO, 1965 ; KLEMENT, 1955). The Japanese isolate was some what related to *P. marginalis* (GOTO, 1965)

P. marginalis and its pvs. cause disease of head lettuce (BURKHOLDER, 1954), lucerne roots (SHINDE & LUKEZIC, 1974) and soft rot of witloof chicory (VAN OUTRYVE et al., 1987). Here we have an instance where cereals have been attacked by isolates of *P. marginalis*.

The PDI values show that IR-36 among rice cvs. and Karan 4 among barley cvs. were most susceptible. The values of r and r_1 were duration - and cv.- specific for rice but not for barley. In all the three cvs. of barley r was uniformly maximum after 10d and r_1 between 5-10d of disease progress (table-2). Such variations in values of PDI, r and r_1 synchornous to host responses were recorded earlier in tomato leaves inoculated with *X.c. pv vesicatoria* (KUMAR & PRASAD, 1987). At penultimamte stage of disease bacterial numbers recovered from rice and barley leaves ranged between 1.92-2.12 $x10^3$ and 1.68-2.02$x10^3$ CFU/ml respectively. The number dropped with spread of laminar necrosis.

Three isolates obtained from rice barley and pea leaves on examinatioin show that the bacterium is gram -ve rod measuring 0.2-0.5 x 1.0 in size with 1-3 polar flagella at one end. Optimum temperature is 26-28 C with 5% NaCl tolerance. On KBA medium greenish blue fluorescence is emitted, and KAA grown culture are creamish yellow turning light brown. Colonies of 48 h are irregular and 3-4 mm in diamemter. The margin is undulate, elevation flat and consistency viscid. Glucose, sucrose, trehalose, B-alanine, l-arginine, sorbitol and 2-keto lactose promote growth in varying degrees.

Table 1- Symptoms progress in rice cvs.IR- 36,Rajendra202 and Mussorie and barley cvs. Karan 4, BAU-2205 and BR-32 after infection of *P. marginalis*.

Days after infection		Symptoms
Rice	5	No infection
	12	light brown small 1-2 spots appear at margins and tips in leaves.
	16	Spots increase to 4-6. Chlorosis with yellow green patches and bleaching of leaf tips begin. Water soaking visible in 4-5 leaves/plant.
	20	Dark brown, oblong spots coalesce to 10-12 big lesion/leaf. Chlorosis and tip bleaching in leaves increase.
	30	Water- Soaking disappears. Lesions number increase to 20-25 /leaf.Yellowing, chlorosis and edge necrosis of larger number of leaves obvious.
	40	Entire inflorescence and a few grains to covered by dark brown to black lesions.
	45	Large scale yellowing and extensive spreadout of lesions apparent on leaves, inflorescence and maturing seeds.
Barley	5	No infection
	10	Yellowing, chlorosis and bleaching in numerous leves start simultaneously at edges and tips, which preceede appearance of light brown (2-3) spots in the centre.
	20	Yellowing and chlorosis progress from tip downwards. Light brown spot 10-12/ leaf arranged lengthwise at the edges and in centre. No water soaking visible.
	25	Spots become dark brown and oblong. Larger number of leaves become chlorotic and bleached.
	30	In two third of the leaves per plant extreme bleaching and chlorosis set in. Long linearly coalescing light brown to dark brown spots 25-30/leaf appear.

Table 2 Progess of disease in rice cvs. and barley cvs. after infection of *P. marginalis.*

Days after infection	PDI			r			r_1		
Rice	IR-36	Rajendra 202	Mussorie	IR-36	Rajendra 202	Mussorie	IR-36	Ranjendra 202	Mussorie
5	-	-	-	-	-	-	-	-	-
12	2.00	1.60	1.40	0.0002	0.4384	0.4505	0.0003	0.6889	0.7080
16	5.50	5.20	4.70	0.1142	0.1455	0.0469	0.4281	0.5494	0.1757
20	11.47	9.80	8.87	0.0069	0.0103	0.0151	0.3290	0.0490	0.0719
30	22.00	21.72	20.0	0.0214	0.2066	0.1920	0.6220	0.5991	0.0588
40	30.05	28.20	26.00	0.0271	0.0315	0.0235	0.1056	0.1231	0.0918
45	40.60	36.95	34.4	0.0230	0.0220	0.2300	0.2023	0.1940	0.2026
Barley	Karan4	BAU-2205	BR-32	Karan4	BAU-2205	BR-32	Karan 4	BAU-2205	BR-32
5	-	-	-	-	-	-	-	-	-
10	3.75	3.00	2.25	0.4661	0.4909	0.5228	0.8390	0.8836	0.9410
20	11.25	10.12	9.00	0.0326	0.0362	0.0400	0.0619	0.0689	0.0760
25	19.37	17.63	16.50	0.0147	0.0165	0.0146	0.0704	0.0793	0.0699
30	32.08	29.08	27.75	0.0150	0.0145	0.0086	0.8720	0.0843	0.0501

PDI = Per cent disease index

r = Rate of disease increase from initial day of infection

r_1 = Rate of disease increase between intervening days.

All the isolates oxidize glucose. Only rice isolate is its fermentor. Only barley and rice isolates cause reduction of nitrate and of sucrose, H_2S production from cysteine, and levan from sucrose. But all the three adduce oxidation of 3-ketolactose, show oxidase, arginine dihydrolase and urease activities, starch hydrolysis, Tween 80 hydrolysis with esterase activity and gelatin liquefaction of stratiform type. None of the isolates show ice nucleastion activity and all fail to evoke hypersensitive response in tobacco leaves. All three affect potato maceration. Rice and barley isolates utilize only m- tartaric acid for alkali production. They produce acid prominently with sucrose, maltose and purple lactose, and in a lesser degree with xylose, fructose, dextrose, starch and glycerol.

In emission of greenish blue fluorescence and in their conformity to LOPAT tests the isolates show the characteristics of fluorescent Pseudomonad (CUPPELS & KELMAN, 1980; PALLERONI, 1984). In use up of maltose, sucrose and purple-lactose (and not lactose) for acid production the isolates conform to the characters of *P. marginalis* and not *P. fluoresencse* (DOWSON, 1957).

Several other feautures tabulated above have been taken as diagnostic properties of *P. marginalis* (SHINDE LUKKEZIC, 1974; FAHY & LLOYD, 1983; LUKEZIC & LEVINE, 1987). Hence the bacterium causing new type of leaf spot disease of rice and barley has been tentatively identified as *Pseudomonas marginalis* (Brown) Stevens

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Relationship between phage sensitivity and pathogenicity in *Xanthomonas campestris* pv. *malvacearum*

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ABSTRACT

Bacteriophages lysing *Xanthomonas campestris* pv. *malvacearum* were isolated from soils of cotton fields with history of high bacterial blight incidence at the Gezira Sheme and Agricultural Research Station (GRS) of Sudan. Phage isolations and typing were according to procedures and media described by CROSSE and GARETTE (1963). Pre and post-Barakat isolates were used as propagating strains and subsequently for the determination of routine and test dilutions (RTD). About 125 isolates from seven cotton varieties were phage typed at the GRS using fourteen phage isolates. The results revealed that the pre- and post-Barakat isolates can be distinguished by their sensitivity to six phages given the numbers 1,2,3,4,6 and 7. This was further confirmed by testing more isolates from other Sudanese varieties and stock cultures of Sudanese isolates at INRA Versailles. Those resembling the pre-Barakat isolates hereafter referred to as Race 1 were found to vary in their sensitivity to the phages rangir from completely insensitive to highly sensitive to some but not all of them while the post-Barakat isolates (Race 2) are sensitive to all the six phages. Generally phages 1,3 and 4 react with the two races while phages 2,6 and 7 lyse only Race 2. Pathogenicity tests revealed that only isolates of the latter phage type are pathogenic to both Acala (susceptible variety) and Barakat (with B2 B6 resistance factors), while the others (Race 1) produce an hypersensitivity reaction on Barakat. Field host range and *in vivo* bacterial multiplication studies, revealed that the two phage groups are quite distinct pathologically. Race 1 infects the pre-Barakat cultivars while Race2 infects all hirsutum and barbadense varieties grown in Sudan. The bacterial growth rates were higher in the congenial combinations associated with water-soaking and lower in the non-congenial conbinations associated with hypersensitivity. Reactions of the differential lines and cultivated Sudanese varieties showed that Race 1 is pathogenic to all varieties showed that Race 1 is pathogenic to all varieties or lines with B2, B3 or B2 B3 resistance factors while Race 2 infects also B2 B6 and B2 B3 B6 lines.

II

Diseases caused by opportunistic and fastidious pathogens. New and unusual diseases caused by prokaryotes

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Diversity in erwinias as plant pathogens

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Fifteen species are now recognised in the genus *Erwinia*, ranging from epiphytes to specialised plant pathogens. Of these, only about a third have been studied extensively because of the importance of the diseases they cause. The plant pathogenic erwinias (12 species) can be divided broadly into two groups, one containing bacteria such as *E. amylovora, E. salicis, E. stewartii* which are not pectolytic and induce wilting and tissue necrosis caused by extra polysaccharides and toxins on a narrow range of plants. The other group comprises the soft rot erwinia such as *E. carotovora, E. chrysanthemi* which are pectolytic, relying mostly on a battery of different pectic enzymes which they produce in large quantities to cause soft rot of parenchymatous tissues and occasionally wilting, also often on a broad range of plants. In contrast to bacteria in the first group, pathogenicity of the soft rot erwinias are markedly affected by environmental factors such as $p.0_2$ and temperature. It is not clear to what extent the apparent narrow host range in the field of certain soft rot erwinias such as *E. carotovora* ssp. *atroseptica* can be attributed to genetic factors or to their geoclimatic distribution and that of potential hosts.

The erwinias are an important group of bacteria which have attracted considerable attention as plant pathogens. The 9th edition of Bergey's Manual (LELLIOTT & DICKEY, 1984) recognises fifteen *Erwinia* species (Table 1). All but two species (*E. uredovora* and *E. herbicola*) are well established plant pathogens.

Of these, however, about a third have been examined to some depth because of the economic importance of the diseases they cause (*E. amylovora, E. salicis, E. stewartii, E. carotovora* and *E. chrysanthemi*). This paper reviews briefly the diversity in erwinias as plant pathogens, focusing on symptoms expressed, host specificity, ecology and pathogenicity. As several aspects related to the subject have already been reviewed (EDEN-GREEN & BILLING, 1974; SCHROTH *et al.*, 1974; PÉROMBELON & KELMAN, 1980; DYE, 1981; PÉROMBELON, 1981,1991; KOTOUJANSKY, 1987; VERDONCK *et al.*, 1987), only key references and those not cited in the reviews will be given.

Symptoms

Table 1 lists the diseases caused by the erwinias, the main or specific hosts and typical symptoms. A wide range of plants, both annuals and perennials, are affected and infection can result in tissue necrosis, wilting of the plant or maceration of parenchymatous tissue. The pathogenic erwinias can be divided into two groups based on symptomatology; one comprises bacteria which cause tissue necrosis or wilting (species no. 1-8) and the other bacteria which primarily cause soft rots although wilt symptoms can be induced under certain conditions (species no. 11-15). Most erwinias can be described as vascular pathogens in the sense that they can be transmitted, albeit sometimes in a limited way, through the vascular system. Only in the soft rotting of storage organs by some erwinias is vascular transmission absent.

As with other plant bacterial pathogens, erwinias usually gain entry to their hosts through natural openings or wounds. *E. amylovora* infects its hosts by several routes, e.g. apple and pear flowers can become infected through stigmas, anthers, calyx lobes and nectarial tissues resulting in fire blight disease, while infection of leaf stomata and lenticels on young shoots as well as wounds caused by hail results in twig blight. Late summer infection often results in canker formation on stems (EDEN-GREEN & BILLING, 1974; SCHROTH *et al.*, 1974). *E. rubrifaciens* which affects Persian walnut (*Juglans regia*) as does *E. nigrifluens* is transmitted by mechanical harvesters. The bacteria invade the tree and

eventually cause necrosis of the bark (AZAD & KADO, 1984). Little is known about the diseases caused by the other necrosis-forming erwinias.

E. stewartii is a typical example of a wilt-causing erwinia (PEPPER, 1967). The bacteria are transmitted by insects and grow in xylem vessels which become blocked by large amounts of extracellular polysaccharides resulting in wilting of the plant. They can also grow in the intercellular spaces of the leaves of corn seedlings causing loss of membrane semipermeability and water soaking symptoms (COPLIN & COOK, 1990). Wilting followed by desiccation are also the main symptoms caused by *E. tracheiphila* on cucumber and *E. salicis* on willow trees, but the economic importance of infection by the latter lies in the discolouration of the wood (watermark disease) (WONG & PREECE, 1973).

The soft rot erwinias produce large quantities of pectic enzymes which macerate and rot the parenchymatous tissue of storage organs but symptoms on a wide range of plants are diverse ranging from soft rot of stems and leaves to stunting, wilting and desiccation. Environmental conditions determine the type of symptoms and a soft rot tends to occur under wet conditions while wilting and desiccation develops under dry conditions, especially in annuals and in young and fleshy parts of perennials. For example, *E. chrysanthemi* causes typical blackleg symptoms (a black stem soft rot) on potatoes under hot and humid (tropical) conditions similar to those of *E. carotovora* ssp. *atroseptica* under cool wet (temperate) conditions but under hot dry conditions less soft rotting and more wilting and desiccation of the leaves occur (PÉROMBELON & KELMAN, 1987). Extensive, spreading soft rot lesions in parenchymatous stem and leaf tissues occur only after infection of the vascular system (PENNYPACKER *et al.*, 1981). The bacteria grow first in the xylem vessels from where they can invade adjacent susceptible tissues. Under dry conditions, the lesions do not spread but wilt symptoms develop caused more likely by plugging of the xylem by bacterial cells and degraded cell wall products rather than by extracellular polysaccharides or wilt inducing toxins. Potato blackleg is somewhat an exception as the bacteria first multiply in the rotting mother tuber before invading the stems. Otherwise, most other host plants not reproduced from a fleshy organ become infected through

Table 1. *Erwinia* spp.: diseases, symptoms and host range

Bacteria	Disease/symptoms	Host range
1. *E. amylovora*	fire blight, necrosis	*Pomoideae, Rosaceae* (apple, pear)
2. *E. tracheiphila*	bacterial wilt	*Cucurbitaceae*
3. *E. mallotivora*	leaf spot	*Mallotus japonicus*
4. *E. rubrifaciens*	bark necrosis	*Jugans regia* Persian walnut
5. *E. quercinia*	oozing of sap	oak acorn
6. *E. nigrifluens*	bark necrosis	Persian walnut
7. *E. salicis*	blight, water mark	*Salix* spp., *Populus* spp.
8. *E. stewartii*	Stewart's disease, wilt	maize
9. *E. herbicola* (*Enterobacter agglomerans*)	epiphyte galls, necrosis farmers lung	wide restricted clinical specimens
10. *E. uredovora*		rust uredospores
11. *E. carotovora*		
subsp. *carotovora*	soft rot	wide
subsp. *atroseptica*	blackleg	potato
subsp. *betavasculorum*	soft rot	sugar beet
subsp. *odorifera*[1]	chicon soft rot	chicory
12. *E. chrysanthemi*	soft rot, wilt	wide
13. *E. cypripedii*	brown rot	*Cypripedium* orchid
14. *E. rhapontici*	crown rot	rhubarb
15. *E. ananas*	soft rot	pineapple
Species *Incertae Sedis*		
E. carnegiena	soft rot	giant cactus

[1] new subspecies proposed by R. Samson (personal communication)

wounds and natural openings.

Distribution and host specificity

Members of the genus *Erwinia* are widely distributed but individual species usually have a restricted geoclimatic distribution which in some cases is related to that of their host. In general, the necrosis and wilt forming erwinias (species no. 1–8, Table 1) tend to be host specific and consequently with a restricted distribution which may be smaller than that of the host species. The host range can be limited to taxonomically related species only as in the case of *E. amylovora* (*Pomoidae* and *Rosaceae*), *E. tracheiphila* (*Cucurbitaceae*), *E. salicis* (*Salix* spp.), *E. quercinia* (*Quercus* spp.) and *E. stewartii* (*Zea mays* and a few related species). Others are apparently even more host-specific; *E. mallotivora (Mallotus japonicus), E. rubrifaciens* and *E. nigrifluens* (*Jugans regia*).

In contrast, the soft rot erwinias (species no. 11–15, Table 1) tend to have a wider host range encompassing taxonomically different species. An extreme case is that of *E. carotovora* subsp. *carotovora* which has a worldwide distribution and an equally wide host range. *E. chrysanthemi* can infect a narrower range of plants more commonly in the tropics than in temperate regions (field and glasshouse crops). Attempts to subdivide this species into pathovars on the basis of isolation and apparent host specificity (DYE, 1981) are not convincing because of cross pathogenicity and the poor relationship between pathogenicity, biochemical characters and serological reactions (DICKEY, 1981; JANSE & RUISSEN, 1988; SAMSON *et al.*, 1990).

The other soft rot erwinias usually infect only one or a few plants as indicated in Table 1. However, this apparent specificity does not imply inability to infect other unrelated plant species as with the necrosis forming erwinias. Most of the soft rot bacteria can rot potatoes as well as several other plants especially when inoculated. For example, *E. carotovora* subsp. *atroseptica*, the typical blackleg pathogen, has been isolated occasionally from naturally diseased calabrese and turnips growing in the vicinity of blackleg affected potato crops in

Scotland. Its apparent specificity to potatoes could be explained in terms of geographical, climatic or cultural constraints on distribution and more specifically to its inability to survive freely in the environment whilst surviving on vegetatively reproduced potato tubers in temperate regions. There is no evidence in support of a special genetically-based association between this bacterium and potato. This kind of association could be transmission of the bacteria. Until more is known on the ecology of the other soft rot erwinias and the epidemiology of the diseases they cause, their apparent host specificity would remain doubtful. Thus, it would be possible for *E. carotovora* ssp. *betavasculorum* to infect crops other than sugar beet in California if introduced elsewhere, provided the bacterium can survive in its new environment. Similarly, the conditions (wounding, high humidity and temperature) found in the production of chicons of chicory (*Cichorium intybus*) exert strong selective pressure in favour of certain strongly pectolytic bacteria able to grow at high temperatures such as *E. carotovora* ssp. *odorifera*, *E. chrysanthemi* and *Pseudomonas* spp. to cause a soft rot (VANTOMME *et al.*, 1989; LE GUERN *et al.*, 1992; R. SAMSON, personal communication). It is possible that changes in cropping pattern could lead to the emergence of new erwinia forms; erwinias with some characteristics of *E. carotovora* sspp. *carotovora* and *atroseptica* can be found in hot climates on less traditional crops (KARNAJARAT *et al.*, 1987).

Ecology and epidemiology

The ecology of the different erwinias is relatively similar. The main difference lies in the different strategies enabling the bacteria to survive from one growing season to the next and these in turn are related to the hosts and the nature of diseases caused. It is likely that in the course of evolution, progressive adaptation to an environment was paralleled by increasing specialisation in pathogenicity.

As most of the diseases caused by necrosis or wilt forming erwinias (species no. 1–8, Table 1) affect aerial parts of the plant, aerial transmission by airborne insects, wind driven rain, rain splash or aerosols is common. The source of the bacteria is not always known but they are unlikely to survive for long freely in the environment. *E. amylovora*, which is the most documented case, is transmitted

either by insects or by wind blown strands of bacterial ooze or by rain splash. The source of the bacteria can be cankers and blighted twigs from the previous year on diseased apple and pear trees or on alternative hosts (hawthorn in Europe and *Pyracantha* spp. in California) as well as newly infected parts. It is likely that diseased perennial host plants are also the main source with other erwinias, *E. rubrifaciens, E. salicis, E. quercinia* and *E. nigrifluens*. As with *E. amylovora* they overwinter probably in infected parts of the hosts and are similarly transmitted.

In contrast, the soft rot erwinias tend to infect short-lived plants, mostly annuals, which are usually fleshy with little lignification, hence susceptible to tissue maceration by pectic enzymes. It is therefore essential for them to be able to bridge the gap between one growing season and the next, especially in regions with climatically defined growing seasons. Not surprisingly, infection is more often than not from non aerial sources. Of the different soft rot erwinias, the ecology of only *E. carotovora* sspp. *carotovora* and *atroseptica* and to a lesser extent *E. chrysanthemi* has been examined in detail (PÉROMBELON, 1991). The bacteria can be transmitted in the case of the vegetatively reproduced crops on the planting material which can be latently infected (seed potato tubers and *E. carotovora* ssp. *atroseptica*) or which become infected when multiplied by taking cuttings (carnations St Paulia and *E. chrysanthemi*). Both air- and soil-borne insects and nematodes can act as vectors, eg stalk rot of corn caused by *E. chrysanthemi* (pv *zeae*) transmitted by stem boring insects; *E. rhapontici* transmitted by the eelworm *Anguillulina dipsaci* to infect rhubarb; *E. chrysanthemi* and *E. ananas* transmitted by ants and other insects to pine apple. These vectors usually pick up the bacteria from diseased host plants, diseased plant debris from previous growing seasons, alternative hosts (weeds) or the soil.

E. carotovora ssp. *carotovora* is widespread in soil, surface water on leaf surfaces and is found on many insects, nematodes and other soil fauna. This is probably a reflection of its wide host range which provides a continuous supply of the bacteria to the different ecological niches. It does not follow necessarily that the bacteria are long living in soil, but rather populations are continuously being replaced as they die out. Nevertheless, they are undoubtedly more resistant to

environmental stress than the two other above mentioned erwinias. Crops in close contact with the soil surface grown frequently in the same field readily become infected, especially under wet conditions with *E. carotovora* ssp. *carotovora*. In contrast *E. carotovora* ssp. *atroseptica* and *E. chrysanthemi* are rarely detected in the environment probably for the reasons discussed earlier, namely poor survival potential and restricted host ranges. In the absence of the relevant information, it can be surmised that the same holds good for the lesser known soft rot erwinias.

Pathogenicity

The types of symptoms caused by pathogenic erwinias suggest that broadly speaking the bacteria fall into two groups; one contains the 'frank' pathogens which have defined host ranges and tend to cause necrotic lesions and wilt symptoms but do not rot plant tissues; in the other group are bacteria which exhibit little or no host specificity and commonly cause parenchymatous tissue maceration, although some wilt symptoms can be induced under special conditions. Erwinias in the first group are nutritionally demanding and are often auxotrophs whereas those in the second group are prototrophs.

The application of molecular biological principles and methodology has greatly improved our knowledge of the nature of pathogenicity in erwinias. The pathogenicity of only five erwinias has been examined in some detail; *E. amylovora, E. stewartii, E. carotovora* sspp. *carotovora* and *atroseptica* and especially *E. chrysanthemi* (KOTOUJANSKY, 1987) and even then the studies are far from being completed. However, these bacteria could be viewed as representatives of the two groups of erwinia pathogens mentioned above.

Pathogenicity determinants in *E. amylovora* fall into broad categories; those that affect pathogenicity *per se* (qualitative) and are plasmid-borne and those that control virulence (quantitative) and are chromosomic. The latter are related to the production of extracellular polysaccharides (EPS; capsular polysaccharides or amylovorin) and the amount produced is affected by the host and is related to the degree of virulence (CHATTERJEE & VIDAVER, 1986). Its main function is to

restrict water transport in the vascular system causing wilting but can also apparently prevent bacterial attachment to host cells thus avoiding the effects of host resistance factors. In addition, two sets of genes in *E. amylovora* have been implicated in hypersensitive reaction (HR) when tested on tobacco, hrp and dsp, and both are apparently needed for disease development in pear tissue (STEINBERGER & BEER, 1988; VANNESTE *et al.*, 1990). In *E. stewartii*, in addition to EPS, a low molecular weight toxin associated with water soaking lesions has been found (COPLIN *et al.*, 1992). However, the bacterium does not induce HR reaction in any host or non host but the gene for low Mr toxin hybridises with *E. amylovora* hrp DNA. Lastly, there is some evidence that siderophore production in relation to iron uptake could be involved in pathogenicity (VANNESTE & EXPERT, 1990).

The soft rot erwinias are characterised by the production of large quantities of a battery of pectic enzymes believed to be their most important pathogenicity determinants (COLLMER & KEEN, 1986). The enzymes enable them to macerate parenchymatous tissues of many plants by degrading cell wall pectic substances leading to cell death and release of nutrients for bacterial growth. It should be stressed that although all soft rot erwinias produce pectic enzymes *in vitro* and/or *in planta*, certain necrosis forming erwinias, *E. salicis, E. rubrifaciens*, can degrade pectic substances without causing a rot in plant tissues.

Four main types of pectic enzymes can be produced, some as multiple isomers; pectate lyase (PL) which is believed to be the most important, polygalacturonase (PG), pectin methyl esterase (PME) and pectin lyase (PNL) (KOTOUJANSKY, 1987). *E. chrysanthemi* produces *in vitro* up to five extracellular PL isoenzymes, *E. carotovora* ssp. *carotovora* and *atroseptica* produce two periplasmic and two secreted PLs. All three bacteria produce one PG, one PME and one PNL. The last enzyme is intriguing as it is produced *in vitro* only after induction by DNA-damaging agents (UV, mitomycin C, naladixic acid) but it is also produced *in planta* (TSUYUMU & CHATTERJEE, 1984). *E. rhapontici* produces only PNL which explains why, although able to rot plant tissues, it is described as non-pectolytic. A similar explanation probably applies to *E. cypripedii* and *E.*

ananas which although can induce a soft rot are not pectolytic on pectate based growth media. In addition to pectic enzymes, siderophore production in relation to iron uptake has also been implicated as with *E. amylovora* in pathogenesis (ENARD *et al.*, 1988). No other pathogenicity determinants such as those found in *E. amylovora* and *E. stewartii* have been clearly identified. However, there is some evidence that as yet uncharacterised cryptic pathogenicity genes expressed only in plants are also present (BEAULIEU & VAN GIJSEGEM, 1990).

There is one aspect of pathogenicity which affects differently the two groups of erwinias; the effect of environmental factors on the expression of pathogenicity determinants especially in relation to competition at the infection court with other erwinias and other saprophytic bacteria also present. The presence of more than one erwinia as well as other microorganisms in latent and active infections is a common phenomenon.

Host plant infection by *E. amylovora* is not markedly affected by environmental factors. Infection occurs when air humidity is high and temperatures are between 18–32°C which allow satisfactory multiplication of the bacteria provided the initial inoculum load is already high (10^4–10^6 cells) (SCHROTH *et al.*, 1974). Insects, wind-driven bacterial ooze and arrested lesions from the previous year can account for the high inoculum level. However, growth of the pathogen on plants can be affected by the ubiquitous leaf epiphyte, *E. herbicola*. This effect has been attributed to an antibiotic which is inhibited by histidine *in vitro*, hence is not readily detected. No doubt other mechanisms are also involved, one of which, competition for nutrients, is perhaps the most likely (VANNESTE *et al.*, 1992). Thus, ERSKINE & LOPATECKI (1975) postulated that growth of *E. herbicola* was favoured with concomitant decrease in growth of *E. amylovora* to a level below that needed for initiation of infection when temperature and humidity decreased and sugar levels in host tissue increased.

In the case of the second group of pathogens, the soft rot erwinias, there is a closer relationship between pathogenicity, host resistance and the environment than with the first group of pathogens typified by *E. amylovora*. Infection is often

latent in the plant and remains so until there is a change in the micro environment which impairs host resistance allowing growth of erwinias. Under these conditions, disease can develop from low numbers (<10^2 cells) of the bacteria. Host induced or active resistance mechanisms are greatly reduced under hypoxic conditions (<2% 0_2) which can develop when tissue respiration rate exceeds oxygen diffusion. This is brought about by high temperatures (>30°C), a water film sealing the affected organ or water congestion of the affected tissue caused by cell contents leakage following infection by, for instance, another pathogen, fungal or bacterial. Under these conditions growth of pectolytic anaerobic or facultative anaerobic bacteria is favoured, especially those producing large amounts of pectic enzymes. The higher the inoculum load, the sooner the critical number (10^8 cells) of erwinias for symptom expression is reached.

Temperature is the main factor affecting the relative virulence of soft rot erwinias and its level may determine which organism predominates in a lesion when more than one form is present. *E. carotovora* ssp. *atroseptica* causes potato blackleg in the field when temperatures are <25°C whereas the opposite is true for *E. chrysanthemi* even when both bacteria are present in equal numbers on the seed tubers (PÉROMBELON *et al.*, 1987). This differential effect of temperature can be explained in terms of the level of production of PL and PG. Enzyme production in *E. carotovora* ssp. *atroseptica* in pectate broth was found to be four times greater at 27°C than at 30°C although total protein production and cell growth were comparable at both temperatures (LANHAM *et al.*, 1990). Similarly, competition, as affected by the effect of temperature on pectic enzyme production and bacterial growth rate, between *E. carotovora* ssp. *carotovora* and *E. carotovora* ssp. *atroseptica* or other pectolytic bacteria in the rotting mother tuber could explain why *E. carotovora* ssp. *carotovora* does not cause blackleg in temperate regions and in some warmer areas. As pathogenicity of many soft rot erwinias is determined mostly by their pectic enzymes which are also produced by many saprophytic bacteria, they can be regarded as being opportunistic pathogens.

Taxonomic considerations

This brief review of the diversity found among plant pathogenic erwinias suggests that the bacteria appear to fall in two broad groups on the basis of symptoms induced, host specificity range, nature of pathogenicity and, to a lesser extent, ecological behaviour. The genus *Erwinia* was initially established arbitrarily for essentially plant pathogenic members of the *Enterobacteriaceae* (WINSLOW *et al.*, 1917). Not surprisingly, most workers who have studied the genus have suggested splitting it into two or more genera and even distributing at least some of the species throughout other well established genera of the family *Enterobacteriaceae* (STARR & CHATTERJEE, 1972). The only proposal which was accepted for some time mostly by bacteriologists outside the USA was that of WALDEE (1945) who restricted the genus *Erwinia* to the non-pectolytic phytopathogenic enterobacteria (species 1-10, Table 1) and grouped the pectolytic phytopathogenic forms in a new genus, *Pectobacterium*. The two genera, *Erwinia* and *Pectobacterium*, are equivalent to the above mentioned two groups, necrosis and soft rot forming erwinias respectively leaving out the non pathogenic erwinias. However, Waldee's proposal never gained universal support and has long been discarded.

Recent phenotypic studies (DYE, 1981; MERGAERT *et al.*, 1984; VERDONCK *et al.*, 1987) indicate that the erwinias could be divided into three groups broadly similar to the *Amylovora*, *Herbicola* and *Carotovora* groups of DYE (1968,1969a,b). The first and last groups are equivalent to the two groups identified in this review with the exception of *E. ananas* which was placed in the second group (*Herbicola*). Taxonomic and nomenclatural problems exist regarding members of the so-called *Herbicola* group; *E. herbicola* is usually known as *Enterobacter agglomerans* by medical bacteriologists and two ill-defined strains (syn. *E. milletae* and syn. *E. gypsophila*), cause galls, one on *Milletia japonica* and the other on *Wistaria* spp. In addition, strains of *E. herbicola* have been reported to be pathogenic to onion (HATTINGH & WALTERS, 1981) and papaya (NELSON & ALVAREZ, 1980; WEBB, 1985) causing leaf necrosis and cankers.

Although considerable information is available on the serology of certain individual erwinia species notably *E. amylovora* and *E. carotovora* which are serologically homogeneous and heterogenous respectively (SLADE & TIFFIN, 1984; DE BOER *et al.*, 1987), the serological relationship between different *Erwinia* spp. have been neglected.

Results of genomic analysis is confusing; on the one hand, GC ratios would redistribute the erwinias in different groups within the genus (STARR & MANDEL, 1974) and on the other hand DNA re-association studies indicate that erwinias are not significantly different from one another as they are to other members of the *Enterobacteriaceae* (BRENNER *et al.*, 1972). The implication would be a general reorganisation of the genus *Erwinia* and no doubt of the *Enterobacteriaceae* too.

Although there is a consensus of opinion about the identity of most species based mostly on physiological characters, there is still some doubts about the taxonomic relationships between species. The question is whether it is justifiable to reclassify erwinias into two or more new genera. It is unwise to rely solely on such criteria as pathogenicity determinants and type of symptoms to define genera. However, these criteria are of importance to plant pathologists and, bearing in mind the arbitrary nature of the grouping of plant-associated enterobacteria in the genus *Erwinia*, should be given more weight in phenotypic studies. Increasingly available information on the degree of DNA homology of the pathogenicity genes should be of value in determining phylogenic relationships of the erwinias. For historical and plant pathological reasons it may be best to retain the separation of plant pathogenic or plant associated species from other genera of the family *Enterobacteriaceae* but there may be a case for reclassifying them into two or more genera.

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Tropical diseases incited by fastidious bacteria

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ABSTRACT. In the last twenty years, fastidious bacteria have been discovered for the first time to cause or at least be associated with numerous plant diseases. Most are tissue specific within their host plants, either inhabiting xylem or phloem. Fastidious xylem-inhabiting bacteria (FXB) have been isolated in axenic culture on media formulated specifically for their growth. Characterization of different FXB has lead to the description of two new genera and species as *Xylella fastidiosa* and *Clavibacter xyli*. Each species contains more than one pathogen. In general, members of *X. fastidiosa* are Gram-negative, insect-transmitted, and limited in geographic distribution to the Americas; whereas, those of *C. xyli* are Gram-positive, mechanically transmitted, and widely distributed throughout the world. Fastidious phloem-inhabiting bacteria (FPB) are generally Gram-negative, insect-transmitted, and occur in different geographic locations throughout the world. FPB have not been grown in axenic culture, except possibly for the citrus greening organism.

This paper addresses various aspects regarding diseases incited by fastidious bacteria including economic importance, distribution, recent research, and need for further research. Emphasis will be placed on diseases of tropical importance with special reference given to ratoon stunting disease of sugarcane caused by *C. xyli* subsp. *xyli*.

KEYWORDS. *Xylella fastidiosa*, *Clavibacter xyli*, fastidious bacteria, plant disease

Although prokaryotes have been recognized as plant pathogens since the pioneering research of Burill in 1881, the concept that some are extremely fastidious endophytes developed only recently following the discovery by Doi *et al.* and Ishie *et al.* in 1967 that phloem-inhabiting mycoplasma-like organisms are associated with certain plant diseases. This discovery inspired further investigations into the etiol-

ogies of numerous plant diseases for which the identity of the causal agents was uncertain. Unexpectedly, not only were additional mycoplasma-like organisms discovered, but so were other fastidious prokaryotes. These include both spiroplasmas, which like the mycoplasma-like organisms lack cell walls, and certain bacteria with cell walls. The prokaryotes in this latter group, especially those affecting plants in tropical climates, are the subject of this paper.

In general, the fastidious bacteria are limited to either the xylem or phloem in the vascular system of their host plants, but a few have been found in both tissues (DAVIS, 1991). Some are transmitted from plant to plant by insect vectors which also serve as hosts (PURCELL, 1982). As a whole, they are phylogenetically diverse and grouped together largely because of their association with plant disease, endophytic habitat, and fastidious prokaryotic nature.

Fastidious, phloem-inhabiting bacteria (FPB) have been found to be associated with at least 15 plant species in widely different areas of the world. They are usually transmitted between plants by phloem feeding leafhoppers or psyllids. Although there have been reports of the *in vitro* cultivation of FPB, these reports have not been unequivocally confirmed. They include the cultivation in chick embryos of a bacterium from yellows-diseased grapevine (NIENHAUS *et al.*, 1978) and the cultivation in axenic culture of the bacterium associated with citrus greening disease (GARNETT, 1985).

No clear taxonomic concept exists yet regarding FPB; however, they appear to have several structural features in common, which may indicate a common phylogeny and eventual recognition as a taxon (DAVIS, 1991). Their cell wall ultrastructure is usually similar to that of Gram-negative bacteria; however, the apparent absence of an R-layer or peptidoglycan layer in the periplasmic space of the cell wall of the citrus greening associated bacterium (MOLL & MARTIN, 1974) may have some significance. Recent studies on the bacteria isolated from citrus greening affected plants indicate a possible relationship to Gram-positive bacteria in the genus *Clavibacter* (HAP, 1991). This would explain the absence of an R-layer but not the presence of an outer cell-wall membrane which is characteristic of a Gram-negative type of cell wall (ARIOVICH & GARNETT, 1985; GARNIER & BOVE, 1977).

The fastidious, xylem-inhabiting bacteria (FXB) include both Gram-positive and Gram-negative organisms. The FXB that causes Pierce's disease of grapevines, almond leaf scorch disease, and alfalfa dwarf disease was the first of the FXB to be isolated in axenic culture (DAVIS *et al.*, 1978). Subse-

quently, all known FXB have now been cultured axenically, and their role as plant pathogens has been conclusively established in many cases.

Most of the Gram-negative, FXB have been found to be related to one another and have been classified as *Xylella fastidiosa* (WELLS *et al.*, 1987). Members of *Xylella* are phylogenetically related to the xanthomonads based on 16s rRNA analysis and belong within the gamma subgroup of the eubacteria. Numerous plant diseases are associated with bacteria classified as *X. fastidiosa* (DAVIS, 1991; HOPKINS, 1989). Some cause leaf scorch diseases of shade trees in less temperate climates of North America including diseases of elm, mulberry, oak, and sycamore. Others including Pierce's disease of grapevines, phony disease of peach, and plum leaf scald are geographically limited to tropical and subtropical climates. Although most of the diseases occur in North America, Pierce's disease of grapevines occurs in North, Central and South America, plum leaf scald occurs in North and South America, and a recently described disease, known as citrus variegated chlorosis (LEE *et al.*, 1991), is presently known to occur only in South America. Diseases associated with some other Gram-negative FXB, such as Sumatra disease of cloves (BENNETT *et al.*, 1987) and a leaf scorch of *Macadamia integrifolia* (JIMENEZ, 1982) occur in tropical regions.

The Gram-positive FXB include *Clavibacter xyli* subsp. *xyli*, the causal agent of ratoon stunting disease (RSD) of sugarcane (DAVIS *et al.*, 1980) and *C. xyli* subsp. *cynodontis*, the causal agent of Bermudagrass stunting diseases (DAVIS & AUGUSTIN, 1984; DAVIS *et al.*, 1983). These two plant pathogens have been found in many areas of the world. They are mechanically transmitted and readily spread in host material used for vegetative propagation. No insect vectors are known. They are related to some other phytopathogenic coryneform bacteria in the genus *Clavibacter* (DAVIS *et al.*, 1984).

Among the fastidious bacterial plant pathogens, *Clavibacter xyli* subsp. *xyli* is one of the most important on a worldwide basis, especially in tropical and sub-tropical climates. Since the identification of *C. xyli* subsp. *xyli* as the causal agent of RSD, considerable information on the host-pathogen interaction has been gained. Recently, the tissue-blot enzyme immunoassay (TB-EIA) was described for detecting vascular bundles colonized by *C. xyli* subsp. *xyli* in sugar cane stalk tissues (HARRISON & DAVIS, 1988). The extent of colonization of vascular bundles is highly related to population density of *C. xyli* subsp. *xyli* within sugarcane plants, and yield reduction due to RSD is directly correlated to

population density (DAVIS *et al.*, 1988a). It follows that yield reduction and the extent of colonization should be correlated. Furthermore, resistance to RSD is inversely correlated to susceptibility to infection (HARRISON & DAVIS, 1986). In a properly structured test, the TB-EIA can be used to obtain data on disease incidence as a measure of susceptibility to infection and on the extent of colonization by the pathogen as a measure of disease severity.

With this knowledge, a method to screen sugarcane for resistance to RSD is now being incorporated into the joint sugarcane breeding program of the U. S. Department of Agriculture, the University of Florida, and the Florida Sugar Cane League at Canal Point, Florida. The utility of the method is based upon a modified TB-EIA designed to accommodate the handling of large numbers of samples. This work began with the assumption that the size of pathogen populations could be use as a criterion for selection in a breeding program because of its correlation with yield reduction. However, some cultivars exhibit tolerance to RSD which limits the correlation (DAVIS *et al.*, 1988b). The ultimate goal in breeding for resistance to RSD may best be to develop high yielding cultivars that will not sustain an epidemic and, thus, have population immunity. This could be accomplished by selection of cultivars for low epidemic potential based on a criterion of incidence and severity without regard to yield reduction. Because of the phenomenon of tolerance, selecting for lower epidemic potential should promote faster progress toward the goal of population immunity than selecting for low yield reduction.

As with RSD, the use of resistant cultivars is the most practical means of control for some diseases caused by fastidious bacteria: however, in other disease situations, resistance is not known to occur or is not feasible to use. Most fastidious bacterial pathogens, unlike *C. xyli* subsp. *xyli*, are transmitted by insect vectors. This complicates matters by adding another dimension to their epidemiology, but also presents the possibility of disease management through vector control. Many strains of *X. fastidiosa* appear to be weak or opportunistic pathogens which cause disease in hosts predisposed by various stresses, and reducing stress might provide a practical means to limit disease in these situations (HOPKINS, 1989). Because of the limited geographic distribution of many of these pathogens, quarantine measures to prevent their introduction and spread into new areas are important.

For the most part, scientific investigations of fastidious bacterial plant pathogens are still in their infancy. Al-

though considerable progress has been made recently, much of it has been descriptive. The fastidious nature of these pathogens still presents barriers to research which need to be overcome. Fortunately, newer technologies will without a doubt provide a means to overcome or circumvent some of these limitations. Technologies, such as monoclonal antibodies and the polymerase chain reaction (PCR) for amplification of specific DNA sequences, should be especially useful for detection and identification of these pathogens and, thus, open the way for research in many areas. With this in mind, the prospects for rapid scientific advancement in studies on fastidious bacterial plant pathogens looks very promising for the future.

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Molecular biological approaches to the identification of mycoplasma-like organisms

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Abstract

Numerous mycoplasma-like organisms (MLOs) are known to be associated with a variety of plant species important in agro-forestry. However, due to the lack of effective techniques for in vitro culture, only limited progress has been made in the identification and classification of these important pathogens. Using recombinant plasmids containing MLO DNA segments of clover proliferation (CP), cloned CP MLO-specific DNA probes hybridized with DNAs isolated from CP MLO-, alfalfa witches'-broom (AWB) MLO- and potato witches'-broom (PWB) MLO-infected plants, but not with DNAs from four other MLO specimens nor with DNA from healthy plants. Using polymerase chain reaction (PCR), the MLO DNA detection sensitivity was increased by at least 10^2- to 10^5-fold over a conventional dot-blot hybridization method. A simultaneous genomic DNA amplification and sequencing procedure allowed direct sequencing of genomic DNA isolated from MLO-infected plants, providing a rapid genomic typing of MLO isolates. The MLO detection sensitivity of PCR was higher than that of the following methods and in the order of PCR > biotinylated riboprobe > biotinylated or radioactive dsDNA probe > radioactive oligonucleotide probe. The results indicated that CP, AWB and PWB MLOs are genetically related and form a group of MLOs distinct from other MLOs.

Keywords: Mollicutes, yellows diseases, molecular hybridization, diagnosis, DNA probe, riboprobe, RFLP, PCR.

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Introduction

Since the first report of DOI et al. (1967), numerous mycoplasma-like organisms (MLOs) are known to be associated with a variety of plant species important in agro-forestry worldwide (HIRUKI, 1988; MARAMOROSCH & RAYCHAUDHURI, 1988; MCCOY et al., 1989). However, little progress has been made on their characterization and classification due to the inability to culture these microbes for comparative studies.

Recent research using cloned chromosomal or extrachromosomal MLO DNA from infected plants or insect hosts has yielded a solid basis for the detection and study of genetic relatedness among MLO isolates (BERTACCINI et al., 1990; DAVIS et al., 1988, 1990; DENG & HIRUKI, 1990a, b; KIRKPATRICK et al., 1987). Thus molecular cloning of chromosomal and extrachromosomal MLO DNA and their application in the detection and comparison of MLO at the molecular level opens a new approach for the classification of these nonculturable mollicutes.

In this paper, our research on mostly Canadian MLOs is reviewed, with emphasis on the use of cloned MLO DNA fragments for molecular hybridization analysis, restriction fragment length polymorphism (RFLP)/Southern-blot analysis, polymerase chain reaction and direct sequencing of genomic MLO DNA based on simultaneous genomic DNA amplification-sequencing for MLO identification.

Plants and MLO Isolates. Clover proliferation (CP) was believed to be distinct from clover phyllody (CPD) and aster yellows on the basis of symptomatology and vector-pathogen-host plant relationships (CHIYKOWSKI, 1965). Western aster yellows (AY27) originally isolated from naturally infected chinese aster, potato witches'-broom, alfalfa witches'-broom and hydrangea virescence were collected in Alberta. Eastern aster yellows (EAY) was a gift from Dr. T.A. Chen, Rutgers University, U.S.A. Except for MLOs in alfalfa and hydrangea, all MLO isolates were maintained on Catharanthus roseus plants by periodic grafting in the greenhouse to new plants which, when fully infected with typical symptoms, served as a source for MLO DNA extraction.

Molecular cloning of CP MLO DNA and screening of recombinants. One hundred and forty-six recombinant plasmids were identified based on agarose gel electrophoresis. Dot-blot hybridization revealed that 16 ^{32}P labelled recombinant plasmids specifically hybridized with nucleic acids isolated from CP MLO-infected plants, but not with nucleic acids from healthy periwinkle plants.

Dot-blot hybridization. Southern-blot hybridizations revealed that each of the 16 recombinant plasmids contained a chromosomal DNA fragment of CP MLO, from which 9 containing CP MLO-specific DNA fragments greater than 1 Kb were selected for the preparation of biotinylated DNA probes. The results of hybridization experiments using

the biotinylated or ^{32}P-labelled recombinant plasmid DNA probes are summarized in Table 1. All the recombinant plasmids reacted with DNAs from CP or PWB MLO-infected plants, but they did not react with DNAs from healthy plants or plants infected by the four other MLOs. The labelled DNAs from CP and PWB MLO-infected plants reacted with all the recombinant plasmids. None of the labelled DNAs from plants infected by the four other MLOs reacted with recombinant plasmids.

Table 1. Dot-blot hybridization between nine cloned clover proliferation (CP) mycoplasmalike organism (MLO) DNA fragments and nucleic acids from healthy and various MLO-infected plants.

Recombinant plasmids[a]	Insert size (kb)	Total nucleic acids[b]						
		H	CP	HV	CPD	EAY	AY27	PWB
pCP57	1.1	-	+	-	-	-	-	+
pCP32	1.3	-	+	-	-	-	-	+
pCP132	1.4	-	+	-	-	-	-	+
pCP92	1.8	-	+	-	-	-	-	+
pCP34	2.5	-	+	-	-	-	-	+
pCP16	2.9	-	+	-	-	-	-	+
pCP118	3.7	-	+	-	-	-	-	+
pCP5	4.1	-	+	-	-	-	-	+
pCP110	5.9	-	+	-	-	-	-	+

[a]Nucleic acids isolated from indicated sources were dot-blotted onto nylon membrane and probed against nine biotinylated cloned CP MLO DNA fragments.
[b]Nine cloned CP MLO DNA fragments were dot-blotted onto nylon membrane and probed against radioactive-labelled nucleic acids isolated from the source plants indicated. H = healthy periwinkle; CP = clover proliferation; HV = hydrangea virescence; CPD = clover phyllody; EAY = eastern aster yellows; AY27 = western aster yellows; PWB = potato witches'-broom. (DENG & HIRUKI, 1991).

RFLP/Southern-blot hybridization. From the 9 recombinant plasmids for dot-blot hybridization (Table 1), four with DNA inserts greater than 2 Kb, namely pCP5, pCP16, pCP34 and pCP118, were selected for RFLP/Southern-blot assays of CP and PWB MLOs. Very similar RFLP patterns were obtained for CP and PWB MLOs when these four recombinant probes were used.

PCR amplification of CP MLO and PWB MLO DNA fragments. Two PCR primer pairs were synthesized according to the sequence of the cloned CP MLO DNA with an A + T content of 80% (DENG & HIRUKI, 1990c).

A 196-bp and a 109-bp DNA fragment were amplified from nucleic acids extracted from both CP and PWB MLO-infected plants. No DNA fragments were amplified from nucleic acids isolated from healthy periwinkle plants or CPD, HV, EAY, AY27 MLO-infected plants (DENG & HIRUKI, 1991). The amplified fragments hybridized to a CP MLO-specific internal probe, indicating specific amplification of MLO DNA from nucleic acids isolated from CP and PWB MLO-infected plants. The 196-bp DNA fragment was

observed on the gel that corresponded to 6 x 10^{-5} ng of template for PCR of not only CP but also PWB MLOs. One-tenth volume of the PCR products from both CP and PWB MLOs was subjected to fourfold serial dilutions and positive hybridization signals were observed even after 4^4-fold dilutions. The 109-bp DNA fragment was observed on the acrylamide gel that corresponded to 6 x 10^{-2} ng of template for both CP and PWB. One-tenth volume of the PCR products for CP and PWB MLO were also subjected to fourfold serial dilutions, and positive signals were observed after 4^4-fold dilutions. When no PCR was applied, a minimum of 2.5 ng of nucleic acid samples from both CP and PWB MLO-infected plants was needed to detect MLOs using a CP MLO-specific internal probe. Nucleic acids from healthy periwinkle, whether subjected to PCR or not, did not show hybridization signals.

Table 2. Detection sensitivities of MLOs associated with CP and PWB by different techniques.

Technique	Immuno*	oligo	dsDNA	dsDNA	ssRNA	PCR
Sensitivity		2.5 ng§	2 ng	2 ng	0.5 ng	25 pg to 25 fg
Radioactive probe	-	+	+	-	-	-
Biotin probe	-	-	-	+	+	-
Difference in fold	0.01	1	1	1	4	100-100,000

+ Yes; - No.
*Reported to be 100 times lower than that of dsDNA probes (BOULTON et al., 1984; BOULTON & MARKHAM, 1985).
§Minimum amount of nucleic acids needed to detect CP MLO or PWB MLOs. (DENG, 1991).

Simultaneous amplification and sequencing of MLO genomic DNA. Several methods are available for sequencing PCR products including direct sequencing (WONG et al., 1987), in vitro transcription-based sequencing (STOFLET et al., 1988), asymmetric amplification followed by sequencing (INNIS et al., 1988), and sequencing by means of linear PCR amplification (LPCR) (MURRAY, 1989). An additional recent introduction is a two step method known as coupled amplification and sequencing (CAS), which selects and amplifies the target DNA in Step I, and completes the sequencing and further target amplification in Step II. Knowing that the first amplification stage of CAS could be eliminated under certain conditions, we developed a simple, single step procedure for simultaneous amplification and sequencing (SAS) of target DNA. Using this simplified procedure, the readable sequence was obtained with field collected MLO-infected samples. We were able to determine the sequence of a segment of chromosomal DNA from CP, PWB and AWB MLOs in total DNA preparations from infected plants using a primer pair designed on the basis of the sequence of a cloned DNA fragment from CP

MLO (DENG & HIRUKI, 1990c). The results confirmed the genetic relatedness of CP and PWB MLOs and identified AWB MLO as another member of this group (results not shown).

Discussion

As shown in this study, as well as in similar work from other research laboratories, molecular biological approaches using recombinant DNA technology have opened a new avenue for more explicit studies of nonculturable mollicutes by offering novel methods for the detection and characterization of MLO in plant and insects (DAVIS et al., 1988; DENG & HIRUKI, 1990a-c; KIRKPATRICK et al., 1987; LEE & DAVIS, 1988).

Our results, that the 9 labelled probes did not hybridize with DNAs associated with CPD, EAY, and AY27, indicate that CP MLO is genetically distinct from CPD and AY MLOs, and confirm earlier observations that CP MLO is distinct from AY MLO and CPD MLO (CHIYKOWSKI, 1965; DAVIS et al., 1990), although all three isolates of MLOs are vectored by the same insect, Macrosteles fascifrons (CHIYKOWSKI, 1965; SINHA & PALIWAL, 1969). The fact that RFLP/Southern-blot hybridizations of MLOs of both CP and PWB yielded very similar results further supports the idea that they are genetically closely related and points to a possibility that they are intimately connected to each other in the disease cycle, alsike clover plants probably serving as an overwintering host of PWB MLO and M. fascifrons as its vector in nature.

Using PCR, detection sensitivity was increased by a factor of at least 10^2- to 10^5-fold over the previously used dot-blot hybridization methods even without consideration of 4^4-fold dilution of PCR products. The detection sensitivity of the different methods is in the following order: PCR>biotinylated riboprobe>biotinylated or radioactive dsDNA probe>radioactive oligonucleotide probe (Table 2). The highest sensitivity was obtained with PCR technique. Furthermore, no labelling and hybridization is necessary if gel electrophoresis is performed and the size of the PCR-amplified product is determined and compared with the size of the expected DNA fragment in the detection of MLOs. For large scale screening of MLOs using PCR, however, cost of the PCR operation should be considered.

The use of SAS for the identification and characterization of targeted DNA segments offers a number of advantages over previously described procedures. The total time from the beginning of SAS procedure to loading the sequencing samples on the acrylamide gel is three hrs, and it provides the sequence as well as the size of the target DNA segment in a single step, using the same amount of genomic DNA as PCR. The

advantage of SAS is its simplicity, the reduced amounts of materials required, and the reduced working time. As a single step process it should be readily automated, providing an ideal method for diagnosis, mutation analysis, genotyping, or taxonomic studies.

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Cultivation of *Xylella fastidiosa* in a chemically defined medium

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ABSTRACT

A chemically defined medium, XF-26, supports the in vitro cultivation of strains of *Xylella fastidiosa* originally isolated from grapes showing Pierce's disease (PD) symptoms and almond exhibiting leaf scorch symptoms. The growth of *X. fastidiosa* in XF-26 is comparable to that in the undefined medium CS20 or PD2. Medium XF-26 supports the primary growth of the bacterium isolated from grape tissues showing PD symptoms as well as CS20 or PD2 does.

INTRODUCTION

Strains of *Xylella fastidiosa*, which are organisms associated with diseases that cause tremendous losses in many economically important plants, including grapevine, alfalfa, peach, plum, almond, elm, sycamore, oak, maple, and possibly citrus (HOPKINS, 1989; HOPKINS *et al.*, 1991) have been grown in media that are supplemented with one or more undefined constituents such as soy peptone, Bacto tryptone, phytone, and yeast extract (CHANG & WALKER, 1988; DAVIS *et al.*, 1978, 1980, 1981; WELLS *et al.*, 1981). These constituents are complex and their exact role or necessary growth factors is difficult to define. No defined medium has been reported for the culture of *X. fastidiosa* strains, even though a formulation, namely amino acid-STABA medium was described by CHEN *et al.* (1982). This amino acid-STABA medium supported the growth of two strains of

X. fastidiosa, namely the PLS (plum leaf scald) and RGW (ragweed stunt) bacteria according to CHEN *et al.* (1982). However, no details were given as to what extent the medium would support the growth of PLS and RGW bacteria or if the medium could support the growth of both bacteria isolated from plant tissue.

Knowing the chemical nature of the cultural medium is necessary for determining the nutritional requirements, metabolic pathways, and biosynthetic capabilities of *X. fastidiosa* and for characterizing *X. fastidiosa* strains. We report here the successful cultivation of strains of *X. fastidiosa* in a chemically defined medium.

MATERIALS AND METHODS

Three strains of *X. fastidiosa* were studied: ALS-BC (ATCC 35870), originally isolated from almond with leaf scorch symptoms, PCE-FG (ATCC 35881), isolated from grape with Pierce's disease (PD) symptoms, and R112V2 isolated from grape with PD symptoms in a Georgia vineyard. All strains were maintained and subcultured weekly in CS20 or PD2 agar medium (CHANG & WALKER, 1988; DAVIS *et al.*, 1980).

A defined medium, designated XF-26, consists of the following ingredients at a concentration of gram per liter: K_2HPO_4, 1.5; $(NH_4)_2HPO_4$, 0.5; $MgSO_4 \cdot 7H_2O$, 2.0; trisodium citrate, 1.5; disodium succinate, 1.5; L-alanine, 0.2; L-arginine, 0.4; L-asparagine, 0.4; L-cysteine, 0.4; Glycine, 0.2; L-glutamine, 2.0; L-histidine, 0.4; L-isoleucine, 0.2; L-leucine, 0.2; L-lysine, 0.2; L-methionine, 0.2; L-phenylalanine, 0.2; L-proline, 0.2; L-serine, 0.2; L-threonine, 0.2; L-tryptophan, 0.04; L-valine, 0.2; phenol red, 0.02; potato starch (J. T. Baker Chemical Co., Phillipsburg, NJ), 2.0; and agar (Difco, Detroit, MI), 15.0. The pH of the medium was adjusted to 6.6-6.7 by adding appropriate amounts of 5N NaOH before the medium was autoclaved for 15 min. The medium was poured in petri dish when cooled to 45-50 C. The XF-26 broth medium was prepared the same way without agar.

The XF-26 agar medium was compared to two undefined media, CS20 and PD2, in supporting the growth of the three strains of *X. fastidiosa*. Both CS20 and PD2 were prepared as reported

previously (CHANG & WALKER, 1988; DAVIS *et al.* 1980). Cells of each of the three strains were collected in CS20 broth individually from a 5-day old CS20 culture plate. The cell suspension of each strain was adjusted to a Klett-Summerson Photoelectric Colorimeter reading of 50. A series of 10-fold dilutions were made to 10^{-6} with XF-26 broth to minimize the carry-over of the undefined components from CS20 broth. A 0.1-ml aliquot from each of three dilutions, 10^{-4}, 10^{-5}, and 10^{-6}, was pipetted onto duplicate plates of XF-26, CS20, and PD2. After 14 days' incubation at 30C, the number of colonies per plate was recorded, and the diameters of 20 colonies on each plate were measured.

The XF-26 was compared to CS20 and PD2 in its ability to achieve isolation of *X. fastidiosa* from tissues of grapevine with PD symptom. Symptomatic leaves from two grapevines (R111V1 and R116V11) with PD and asymptomatic leaves from two other grapevines (R57V16 and R117V3) were collected on August 12, 1991. Samples were placed in plastic bags and kept in an ice cooler when shipped from field to laboratory. The cooler containing collected samples was kept in cold room (5C) for 2 days when isolation of the bacterium was attempted. Seven to 10 petioles from each grapevine sample were surface-sterilized in 1.06% sodium hypochloride for 15 minutes, rinsed three times in sterile distilled water, and air-dried under a Laminair flow hood. The sterilized petioles were cut as finely as possible into 3 ml of XF-26 broth medium. The suspension was mixed and streaked onto duplicate plates of XF-26, CS20, or PD2 medium and incubated at 30C. The plates were observed for colony development at weekly intervals using a binocular microscope.

RESULTS AND DISCUSSION

The number of colonies per plate from 10^{-4} dilution was too numerous to count whereas those from 10^{-6} dilution were often too sparse to count. Therefore, the number of colonies and the diameter of 20 colonies were recorded and measured from plates of 10^{-5} dilution. The average number of colonies of strain PCE-FG in XF-26, CS20, and PD2 was 213, 95, and 259 respectively, of strain ALS-BC was 131, 330, and 226 respectively, and of

strain R112V2 was 325, 275, and 145 respectively. The mean diameter (in mm) of a colony of PCE-FG in XF-26, CS20, and PD2 was 0.66, 0.72, and 1.09 respectively, of ALS-BC was 0.22, 0.6, and 0.42 respectively, and of R112V2 was 0.64, 0.95, and 0.78 respectively. There were differences in growth among three strains in the three media. For example, PCE-FG grew best in PD2, ALS-BC in CS20, and R112V2 in XF-26. All three strains have been subcultured for more than 20 passages in XF-26 with no change in growth rate.

Colonies of *X. fastidiosa* were visible in all three media when suspensions from symptomatic petioles of R111V1 or R116V11 were used, whereas no colonies developed in any of the three media when suspensions from asymptomatic petioles of R57V16 or R117V3 were used as inoculum. The colonies were opalescent white and grew 0.1-0.35, 0.04-0.43, and 0.08-0.40 mm in XF-26, CS20, and PD2, respectively after 7 days incubation. XF-26 medium has been successfully used to isolate *X. fastidiosa* from more than 25 different grapevine tissues with PD symptoms since.

Both XF-26 and PD2 contain trisodium citrate and disodium succinate which are likely to be the energy source for *X. fastidiosa* because no growth was observed when both tricarboxylic acids were omitted in XF-26 (C.J. CHANG, unpusblished). The requirement of tricarboxylic acid for their growth may suggest that *X. fastidiosa* possess the Krebs cycle for energy.

Of the 17 amino acids included in XF-26, glutamine and histidine when incorporated in CS20 promoted growth of strains of *X. fastidiosa*, such as strains that cause phony peach disease, plum leaf scald, and oak leaf scorch disease (C. J. CHANG, unpublished). Whether all 17 amino acids are required by *X. fastidiosa* for growth warrants further investigation. CHEN *et al.* (1982) stated that the PD bacterium and ELS (elm leaf scorch) bacterium have basic growth requirements for as few as nine amino acids, a supplementary source of soluble iron, trace concentrations of minerals (available as contaminants in agar), and growth factors. Deletion of one amino acid or of the iron supplement would reduce growth by 75% or more in the first passage on defined medium, and would

prevent growth in subsequent passages. With the availability of XF-26 medium, the role of essential amino acids for growth of *X. fastidiosa* can lead to studies on the interconversion between tricarboxylic acids and amino acids. The metabolic pathway(s) of this newly named bacterium (WELLS *et al.*, 1987) can be defined in greater detail.

Of the four available undefined media (CHANG & WALKER, 1988; DAVIS *et al.*, 1978, 1980, 1981; WELLS *et al.*, 1981), hemin chloride is included in CS20, PW, and PD2, whereas ferric pyrophosphate is the ingredient in BCYE. DAVIS *et al.* (1980) reported that hemin chloride is not essential for growth of PD bacterium, but hemin chloride enhanced growth of the bacterium. It is, however, obvious that *X. fastidiosa* associated with PD requires no hemin chloride or ferric pyrophosphate for in vitro growth because the PD bacteria grew as well in a medium with or without hemin chloride. Whether hemin chloride or ferric pyrophosphate is required for the isolation of other strains of *X. fastidiosa*, such as those associated with phony peach disease, plum leaf scald, oak leaf scorch, and periwinkle wilt is unknown. Two strains of *X. fastidiosa*, PWT-22 (ATCC 35878, originally isolated from periwinkle showing wilt symptoms) and oak (ATCC 35874, originally isolated from red oak showing leaf scorch symptoms), have grown in XF-26 for more than 15 passages to date (C. J. CHANG, unpublished), indicating that both strains are able to grow in a medium without the incorporation of hemin chloride or ferric pyrophosphate.

The incorporation of starch, bovine serum albumin, or acid-washed activated charcoal into the undefined media contributed to the successful isolation or culture of most strains of *X. fastidiosa* (CHANG & WALKER, 1988; CHEN *et al.*, 1982; DAVIS *et al.*, 1978, 1980, 1981; WELLS *et al.*, 1981). FEELEY *et al.* (1979) successfully isolated *Legionella pneumophila*, a fastidious Gram-negative rod infectious to man, by adding charcoal additives to control the inhibitors in host tissue extracts. The accessibility of XF-26 would direct us to elucidate the role that starch plays in the primary isolation and culture of *X. fastidiosa*.

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Considerations on the appearance of *Pseudomonas corrugata* as a new plant pathogen

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ABSTRACT

Pseudomonas corrugata Roberts and Scarlett was first reported as a tomato pathogen in 1978. In the following ten years, surprisingly, "tomato pith necrosis", the syndrome incited by such a microorganism, was found present in several occasions all over the world, in both glasshouse and open-field conditions. Bearing in mind that the typical "tomato pith necrosis" is characteristically different from similar fungal and bacterial stem diseases and that such a syndrome has not been reported in past authoritative guides on tomato diseases, some questions naturally arise from a phytobacteriological point of view. Has *P. corrugata* always existed or has it evolved from some other related phytopathogenic pseudomonads? In the first case, what was (were) the selective pressure(s) that allowed this soil microorganism to act as a pathogen? Considerations are given on the introduction of new high-yielding cultivars with resistance to diseases as well as new agronomical practices favouring "low-grade" pathogens. Speculations on the possibility of acquisition and exchange of new genetic features among bacterial microflora as well as the man-guided evolution of new pathogens are discussed.

Keywords: Tomato pith necrosis; *Pseudomonas corrugata*

- What is "tomato pith necrosis"?

"Tomato pith necrosis" (TPN) is the name commonly used to indicate a pathological syndrome referred to several *Pseudomonas* spp. (i.e. *P. corrugata*, *P. cichorii*, *P. viridiflava*, *P. fluorescens*) and also to *Erwinia carotovora* subsp. *carotovora* and *E. chrysanthemi* pv. *chrysanthemi*. However, a complete description of TPN should also take into

consideration symptoms on the stem, petioles, leaves and fruits. Consequently, according to the original description given by SCARLETT *et al.* (1978) and to a number of studies carried out on this disease over several years (MARTINS & JACOB, 1987; NAUMANN *et al.*, 1989; SCORTICHINI, 1989), typical TPN is characterized, other than by the necrosis and hollowing of the pith, by dark brown to black stem, petiole and fruit stalk lesions, chlorotic leaves, long adventitious roots sometimes associated with longitudinal stem cracking, no lesions on fruits and leaves and, in the author's opinion, neither stem soft rotting nor leaf spotting. Such a syndrome is typically incited only by *P. corrugata* Roberts and Scarlett. In fact, *P. cichorii*, also causes lesions within the fruits and no adventious root formation, whereas *E.c.* subsp. *carotovora* and *E.c.* pv. *chrysanthemi*, *P. fluorescens* and *P. viridiflava* induce wilting and soft rot of the stem and the latter can also incite leaf spotting. These facts are also confirmed by isolations from diseased plants showing all of the symptoms mentioned, yielding pure cultures of *P.corrugata* on the media and by pathogenicity tests carried out with such cultures. The syndrome is also different from other well-known fungal and bacterial stem diseases, such as *Fusarium* wilt, *Verticillium* wilt, bacterial canker induced by *Clavibacter michiganensis* subsp. *michiganensis*. Concerning pith symptoms, some similarities are shared with bacterial wilt incited by *Pseudomonas solanacearum*. Indeed, when a tomato stem is affected by such a pathogen, the pith appears darkened and subsequently decays. However, other facets of the disease are clearly different, particularly the rapid wilting of the plant. In addition, bacterial wilt is typical of tropical areas, whereas *P. corrugata* displays its pathogenic behaviour especially in temperate and cool areas, more often during spring. Actually, the symptoms nowadays characterizing the typical TPN are not reported in past authoritative guides about tomato diseases (MC KAY, 1949; DOOLITTLE *et al.*, 1961). In addition, expert tomato growers are well acquainted with this disease over the last ten years, when outbreaks induced by *P. corrugata* have been widely reported both in open-field and in glasshouse conditions; sometime it has even been found in cultivations grown for the first time in the area. Consequently, it is possible to argue that this syndrome is really a new tomato disease appeared, probably, during the 1970's.

-What do we know about it?
Evidence exist that such a microorganism is a soil inhabitant (ROBERTS & BREWSTER, 1991 ; KOVACEVICH & RYDER, 1991) and that it can survive in soil for tomato transplanting (SCORTICHINI, 1989) as well as in

irrigation water (SCARLETT *et al.*, 1978) and on alfalfa roots (LUKEZIC, 1979). It produces a toxin involved in the pathogenesis (CHUN & LEARY, 1989) and, in addition, such a bacterium has proved inhibitory to root growth of wheat (ROBERTS & BREWSTER, 1991). On the other hand, it has been found effective as a biocontrol agent against some of the take-all pathogens (KOVACEVICH & RYDER, 1991). Incidence of the disease in the cultivation is usually low, although up to 5-6% of plants have sometimes been found affected by TPN (SCORTICHINI, unpublished results). Interestingly, *P. corrugata* has also been found in pepper (M. LOPEZ, personal communication). Finally, several features useful for pointing out the variability within this species have been assessed (SCORTICHINI *et al.*, in preparation). While waiting for other epidemiological studies (i.e. how *P. corrugata* gains entry into the plant, since no symptoms are observed in roots and the pith at the collar level is very often necrotized), some questions and considerations on the sudden appearance of this new disease seem worthwhile. Has *P. corrugata* always existed or has it evolved from some other related phytopathogenic pseudomonads or from taxonomically unrelated species? The sudden appearance of the disease may be explained theoretically by all the hypotheses. In fact, the acquisition of a genetic trait from a phytopathogenic species conferring phytopathological attitudes to another species may be conceived by bearing in mind all of the genetic exchanges and rearrangements among the bacterial species (see ahead). However, even if such exchanges do exist, the contemporary acquisition all over the world of such features by the microorganism(s) that has subsequently become *P. corrugata* seems difficult to accept. In any case, new selective pressures enhancing the pathogenic activity of the ever-present *P. corrugata* or of the "new" *P. corrugata* are required.

- Why TPN? Man-guided evolution of plant and pathogen.

The updating of tomato cultivation has been very rapid during the last 20 years. Concerning the tomato breeding for processing, according to TIGCHELAAR (1990): "past contributions from plant improvement to the improved efficiency of processing tomato production have resulted from carefully planned programs to <u>redesign varieties</u> to resolve specific production problems or to fit <u>new system of crop culture and management.</u> The use of host resistance to facilitate control of problem diseases and the <u>extensive change in plant architecture</u> to accomodate machine harvest are well documented examples of the important contribution plant breeding has made". In addition, according to RICK *et al.* (1987): "the tomato is a

classical example of the use of wide crosses for cultivar improvement. Such crosses were largely stimulated by the relative lack of variation in cultivated types, particularly prior to 1940". Concerning tomato for fresh consumption according to RICK (1986): "amongst the various categories of improvement disease resistance has been emphasized to the greatest extent.

From the foregoing it seems clear that the cultivars and system of tomato culture have changed a lot in recent years. What relationships might exist between these new acquisitions and the appearance of *P. corrugata*? It is well known that TPN is enhanced by excessive fertilization and irrigation. The new hybrid cultivars utilized in the production for the fresh consumption and which are resistant to some fungi and viruses (i.e. *Fusarium*, *Verticillium*, TMV) are less productive, consequently, to reach a high yield, they need more nitrogen and potassium (up to 500 nitrogen units/hectar and 250 potassium units/hectar per year), which, generally favours plant pathogens. In addition, these new systems of tomato cultivation require auxins for improving the fruit set. On the other hand, particularly during spring and in glasshouse cultivation, large day-night temperature excursions frequently occur which can induce stress in the plant (TOGNONI, 1990) during a period when fruits are draining nutrients from the stem. In fact, it has been noted that the pathogenic activity of *P. corrugata* is higher in just such a period. The relationship between the environmental stress suffered by the new tomato cultivars and the occurrence of TPN is well documented (MARTINS, 1989; JACOB & MARTINS, 1990). *P. corrugata* migth, in fact, be a "low-grade"pathogen needing such predisposing factors within the plant to exert its pathogenic behaviour. Moreover, what do we know about the physiological modifications induced in the plant when a new gene is introduced and expressed? (i.e. *rin* gene conferring the "long-shelf life" character on tomato fruits by slowing down their ripening, and, thus, facilitating their transport). Might these genetic introductions, aimed at increasing the variability of the crop, favour the pathogenic activities of otherwise saprofitic bacteria? Might *P. corrugata* exploit new cultivars and changed systems of cultivation in other crops?

- Genetic exchanges and rearrangements.

Concerning the possible relationships with other bacterial species one could hyphothesise that *P. corrugata* is *P. solanacearum* adapted to temperate areas. Both species induce necrosis on pith and incite incipient adventitious root formation along the stem, even if the latter species causes rapid wilting of the plant. However, DNA-RNA hybridization analysis and

fatty acid profiling of the two species has shown clear differences between them. But if "genes required for pathogenicity and hypersensitivity are conserved and interchangeable among pathovars of *Pseudomonas syringae*", could *P. corrugata* have acquired from *P. solanacearum* only the genes for necrotizing the tomato pith, keeping other features of their own? Plasmid transfer from *Pseudomonas fluorescens* to other *Pseudomonas* spp. as well as to other bacterial genera living in soil has been demonstrated (SMIT *et al.*, 1991), but this implies that the species have to live in the same environment, which is in disagreement with the ecological niches occupied by the two species (tropical vs temperate areas) and with the contemporary appearance of the disease all over the world. However, this mechanism of transfer of genetic traits among different bacterial genera inhabiting the soils is very attractive in attempting to understand how new plant diseases can appear. In addition, if we accept the hypothesis that "perhaps it would be best to think of the bacteria as constituting one gene pool from which any "species" may draw genes as these are required" (HEDGES, 1972), transpositions, replicon exchanges and selection, activation of cryptic genes (i.e. phenotipically silent DNA sequence capable of activation as a rare event in a few of a populations), and periodic selection (i.e. the periodic invasion of an asexual pupulation by clones of higher fitness) (BENNETT & RICHMOND, 1978; YOUNG, 1989) are other paths to follow in trying to understand the evolution of phytopathogenic bacteria. However, speculations apart, what we really know is that *P. corrugata* is a new pathogen that has adapted to the new systems of cultivation. If we accept that plants do not evolve to sustain intensive agriculture, we can conceive that microorganisms can exploit the new ecological niches offered by such man-made crop plants.

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Elisa detection of MLO antigens in flavescence dorée affected grapevine leaves

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ABSTRACT

Diagnosis serological tests, ISEM, ELISA and immunolabelling were designed during the last 10 years in our laboratory for Flavescence dorée MLO. They were efficient on infectious leafhopper vectors and on infected broadbeans. On diseased grapevine plants, irregular results were obtained on the grown in the greenhouse. A lot of work was made during the last two years to apply ELISA to grapevine samples harvested in the vineyard.

The difficulties arose from different aspects of MLO infection in woody plants : low concentration of MLO in the infected cells, scattered spatial distribution and also from the biochemical properties of grapevine tissues.

In this report, we discuss the best way of sampling and the best sampling period for grapevine tissues. We also discuss the extraction medium suitable for grapevine and the possibilities to concentrate the antigens for ELISA.

KEYWORDS

ELISA detection, Flavescence dorée, grapevine.

INTRODUCTION

FLAVESCENCE dorée (FD) is the most important MLO disease of grapevine. An efficient serological test is necessary for epidemiology and for assay scion and rootstock clones.

Diagnosis serological tests were formerly developed in our laboratory. They were efficient to detect FD in vectors and herbaceous test plants, but not yet in grapevine samples from the fields.

The difficulties arose from different aspects of MLO infection in woody plants : low concentration of MLO in the infected cells, scattered spatial distribution and also from the biochemical properties of grapevine tissues.

In this paper we report the results about the best plant part and the best extraction procedure for FD grapevine testing.

METHODS

- Extraction in various media of a lot of different samples of FD infected grapevines.
- Assay in an indirect sandwich ELISA where successive layers are :
  1) Polyclonal antibodies from rabbit
  2) Plant extract
  3) Monoclonal antibodies from mouse
  4) Alkaline phosphatase conjugate from goat
  5) PNPP

RESULTS

I Choice of grapevine samples.

The veins or leaves showing strong FD symptoms give the best results in ELISA tests. (Table 1)

Table 1. Choice of the best grapevine sample.

	Cane phloem		Petioles	Whole leaves	Veins	Lamina without the veins
	Macerat	Extract				
OD Diseased	0.044	0.046	0.051	0.107	0.107	0.072
ODD/ODH			NS*	2.02	1.65	NS*

*NS = non significative
ODD/ODH = ratio OD diseased sample / OD healthy sample

The level along the canes giving the higher results is changing. (Fig. 1)

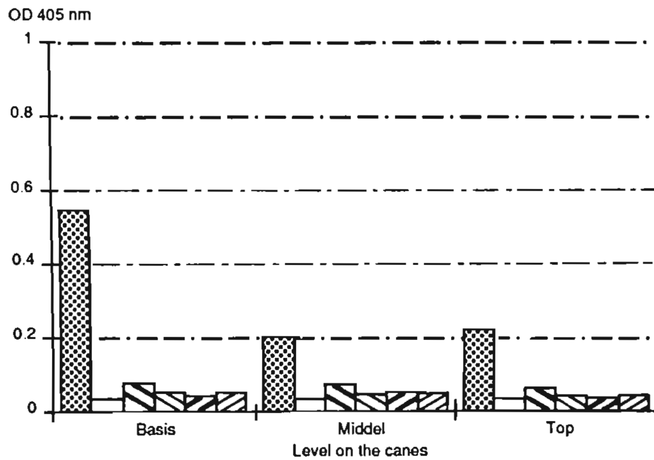
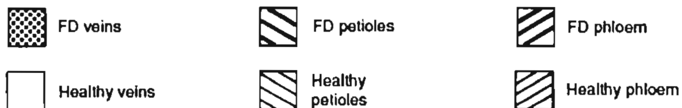


Figure 1. Results of the samples from different levels of the grapevine canes.



II Successive improvements of the extraction medium. Interest of the use of detergents.

The addition of the detergent Triton X 100, then of Chaps was the decisive steps to get clear cut results on the sensitive *Vitis vinifera* scion varieties. (Fig 2)

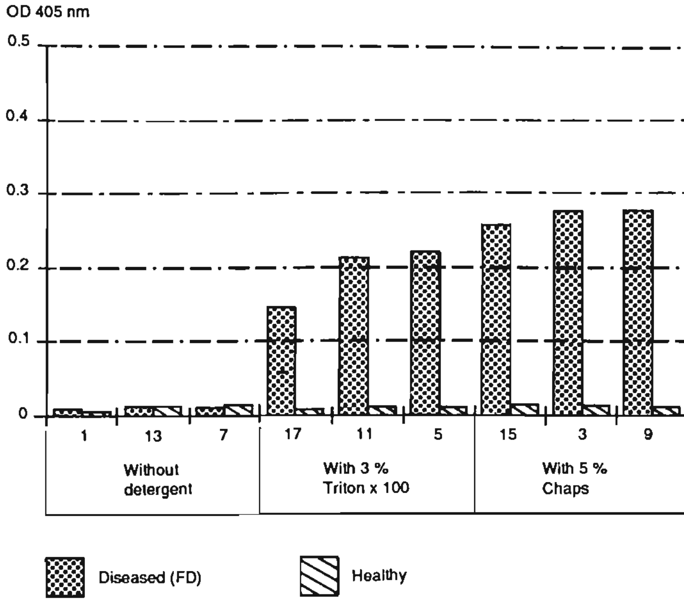


Figure 2. Comparison of 3 different media, used without or with a detergent for extraction of grapevine leaves.

Medium F (Tris : 0.25 M ; NaCl : 0,8% ; Ascorbic acid : 0,53% ; Polyclar AT : 4% ; Thimerosal : 0,02% ; Chaps : 5% ; pH : 8.2) was the best found for MLO antigen extraction from grape tissues.

III Effect of antigen concentration by molecular filtration.

Antigen concentration by molecular filtration, before testing, give an important improvement of the results in grapevine extracts in detergents. (Fig. 3)

For concentration by molecular filtration we used a centrifugation either with centrisart module Sartorius, molecular weight cutoffs 20.000 daltons, or with centriprep 30 module Amicon, molecular weight cutoffs 30.000 daltons. (Fig. 3 and 4)

However detergents and subsequent antigen concentration by molecular filtration decrease sharply the results on FD infected faba bean. (Fig. 4, compare to fig. 3)

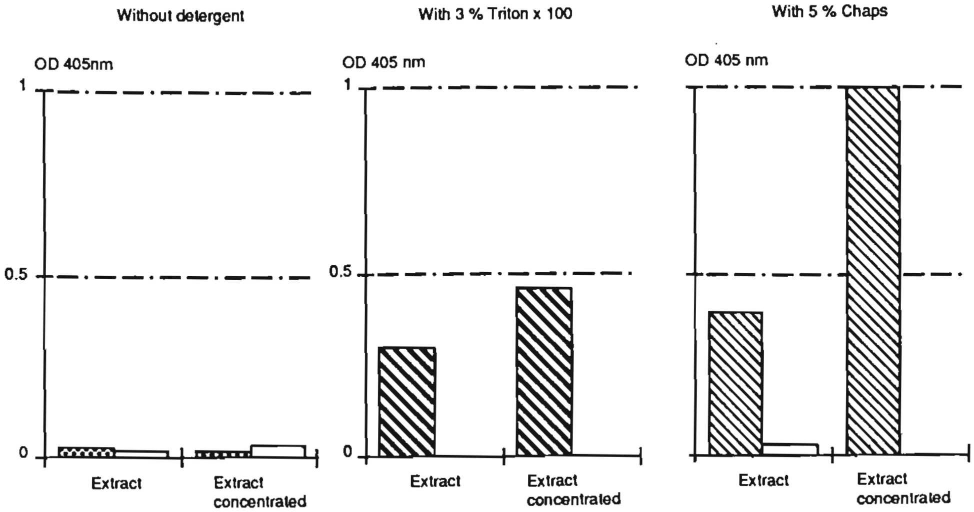


Figure 3. Effect of concentration by molecular filtration on grapevine extracts.

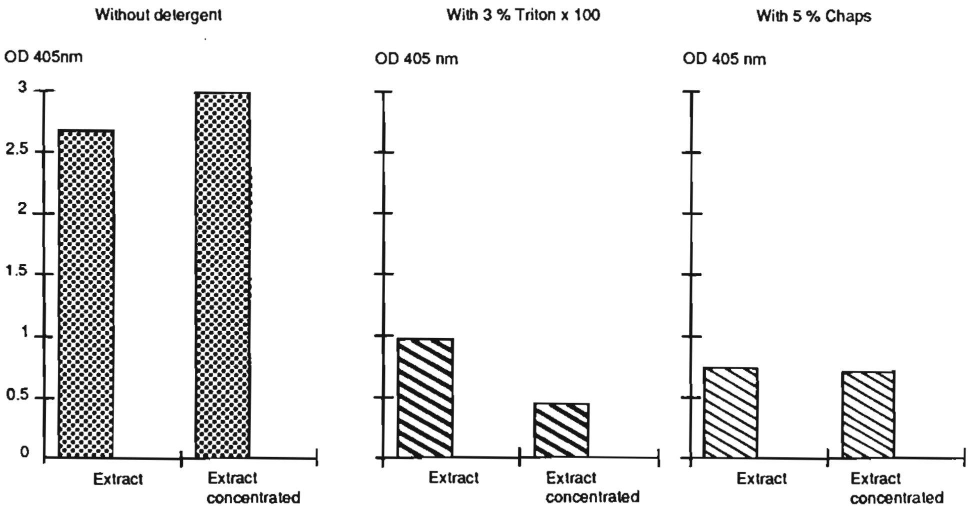


Figure 4. Effect of concentration by molecular filtration on Faba bean extracts.

CONCLUSIONS

- Using our best extraction medium F, containing Chaps 5 %, the ELISA test is effective for detecting most of the FD infected *Vitis vinifera* leaves (Table 2, left).

- Concentration of antigens by molecular filtration before testing improves the results for *Vitis vinifera*. However it is not yet sufficient for testing the infectious symptomless rootstocks. (Table 2, right).

- The improvements obtained using detergent and antigen concentration for detection of FD in grapevine (Figure 3) are not observed in Faba bean extracts where the ability to detect FD decreases sharply when this method is used (Figure 4).

Table 2. Improvement of the results of various samples of grapevine by detergents and molecular filtration; it is not yet sufficient for rootstocks.

Samples and dates	Medium F	
	Extract	Concentrated
10_07_91		
Alicante Narbonne		
Plant n°1	+	+++
n°2	+	++
Carignan Sigean		
Plant n°1	+	++
n°2	++	+++
Grenache Narbonne		
Plant n°1	-	+
n°2	+	+++
n°3	+	++
Rootstock 3309	-	-
4_09_91		
Carignan Narbonne		
Plant n°1	+	+++
n°2	+	+++
n°3	+	+
n°4	+	+++
Grenache Narbonne		
Plant n°1	-	+
n°2	-	+
n°3	-	+
Rootstock 3309	-	-
26_09_91		
Alicante Narbonne	+++	+++
Grenache Narbonne	-	+
Rootstock 3309	-	-

+ : DOM/DOS>2; ++ : DOM/DOS>10; +++ : DOM/DOS>20

DISCUSSION

Use of detergent improves the detection of MLO antigen from grapevine. Results indicate that it may be easier to extract the constituent proteins than intact membranes which are apparently adsorbed on plant cellwall fragments (Figures 2 and 3).

However the antigens associated with membranes may cause an amplification of the ELISA reaction, which does not occurs when membrane is dissolved by detergents (Faba bean extracts, figure 4).

Consequently the detergents favour the extraction of MLO antigen from grapevine tissues but reduce the performance of the ELISA test.

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A bacterial bark canker of alder in Italy

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INTRODUCTION

A bacterial disease of black alder (*Alnus glutinosa*) and Italian alder (*Alnus cordata*) has been observed in several locations of Tuscany and Liguria (Italy) over the last few years.

The disease, which has been given the name of alder bark canker, is characterized by the formation of dark brown necrotic areas, often longitudinally elongated (up to 15-20 cm), in the bark of the trunks but also of the branches, twigs and suckers. A dark watery exudate often oozes from small cracks in the cankers and stains the bark surface (Fig.1).

The necrosis first occurs in the cortical tissue but eventually spreads to the cambium and even the first layers of the wood. Affected trees may die in a few years.

The casual agent is here shown to be a bacterium that appears to be a new species of *Erwinia*.

MATERIALS AND METHODS

Isolations and inoculation experiments

The pathogen was readily isolated from the margin of cankers using nutrient-sucrose (5%) agar medium. On this medium, the pathogen was consistently isolated in pure culture, yielding colonies that were radially striated, low domed, mucoid, with smooth surface and 1-1,5 mm in diameter. A pathogenicity test was developed that involved inoculating two-year-old seedlings of *Alnus glutinosa*, *Quercus robur* and *Juglans regia* with a bacterial suspension of $2x10^8$ cfu/ml. Inoculation was in spring by infiltration of the inoculum into the cork tissue.

Bacterial isolates and bacteriological methods

Six representative isolates, obtained from typical cankers in Liguria (isolates PVFi23, PVFi25 and PVFi27) and in Tuscany (PVFi20, PVFi21 and PVFi22), and six reisolates were examined. Cultures of *E. nigrifluens* (NCPPB564), *E. quercina* (NCPPB1852), *E. carotovora* subsp. *carotovora* (NCPPB2577) and *Pseudomonas syingae* pv. *syringae* (Y32) were examined for comparison.

Morphological, physiological and biochemical characters were examined following methods reported in the "Laboratory Guide for Identification of Plant Pathogenic Bacteria" (1988) and "Manual of methods for General Bacteriology" (1981).

Figure 1. Trunk of an alder tree affected by bacterial canker. The most striking symptom is production of a black exudate that flows down the trunk.

RESULTS

Pathogenicity tests

All six alder bark canker (ABC) isolates produced slightly depressed, necrotic lesions at the site of inoculation within 6-10 days. With time the lesion extended radially and down into the wood and a copious dark-coloured plant sap often leaked

out of the inoculation injuries and stained the surface of the bark. Droplets of a viscous whitish exudate also formed at the site of inoculation. The pathogen was isolated from this exudate. Reisolation from infected tissue yielded the original bacterium.

Characterization of the alder bacteria

ABC isolates were Gram-negative, non sporing rods, motile by means of peritrichous flagella, anaerobic facultative, catalase positive, and oxidase negative. All strains were positive for H_2S from cysteine ,Voges-Proskauer and methyl red tests, acid production from glucose, galactose, fructose, mannose, xylose, arabinose, sucrose, maltose, salicin, glycerol and mannitol, but negative for indole production, arginine dihydrolase, phenylalanine deaminase, lysine and ornithine decarboxilase, acid production from lactose, melibiose, sorbitol and adonitol. Results of other tests are given in Tab. 1.

Table 1 - Comparison of the alder bark canker isolates with reference strains of *Erwinia* spp. and *Pseudomonas syringae* pv. syringae

TESTS	Alder isolates	E. quercina	E. carotovora	E. nigrifluens	P.s. pv. syringae
Growth on MS medium	+	-	+	±	-
ß - Galactosidase	-	-	+	-	-
Tabacco hypersensitivity	-	-	±	-	-
Potato rot	-	-	+	-	-
Pectate liquefaction	-	-	+	-	-
Growth factors requirements	-	+	-	-	-
Cellulase	+	+	+	-	-
Growth at 36 °C	±	-	-	±	-
Hydrolysis of gelatin:	-	-	+	-	-
Acid production from:					
Arabinose	+	-	+	+	+
Melibiose	-	±	±	+	+
Salicin	+	+	±	+	-
Inositol	-	ND	+	+	+

ND = not determined; + = positive result; - = negative result; ± = weak result

CONCLUSIONS

The genus *Erwinia* includes Gram-negative, non-spore forming, oxidase negative and facultatively anaerobic bacteria. The alder pathogen examined here possessed these *Erwinia* characteristics.

The inability to degrade pectate would place the ABC isolates in the *E. amylovora* group.

The symptoms of bark canker noted on *A. glutinosa* and *A. cordata* here were the same as those described by MORIONDO in 1958 and resemble those caused by *E. nigrifluens* on walnut (WILSON et al., 1957) and by *E. quercina*

(HILDEBRAND & SCHROTH, 1967) on oaks. However, these latter bacteria failed to produce any symptoms on alder in inoculation tests.

With regard to phenotypic characteristics, although the ABC isolates shared many characters with *E. nigrifluens* and *E. quercina*, there were a number of differences, particularly as regards acid production from carbohydrates. It is therefore proposed that the ABC organism should be recognised as a new species of *Erwinia*.

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Diversity of bacteria contributing to the decay of ready-to-use salads

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Pseudomonas viridiflava and biovars of *P. fluorescens* have been implicated in the decay of ready-to-use (RTU) salads during storage, but we have observed decay of RTU scarole (*Chicorium endivia*) in the absence of detectable populations of these bacteria. Pectolytic *Erwinia* spp. are rarely found on RTU scarole, and we suspect that normally saprophytic organisms may contribute to the decay of this fragile product. To identify microorganisms involved in decay of RTU scarole, we have attempted to isolate representatives of practically all culturable microorganisms associated with commercially available sachets of scarole. Homogenates of individual leaf pieces were treated and incubated under conditions for isolation of anaerobic spore-forming, aerobic and microaerophilic prokaryotes as well as yeasts, filamentous fungi and protozoa that might be associated with plant material. About 1200 isolates representing over 400 types of organisms distinguished by colony morphology were purified and are being identified. From 64 leaf pieces of different origins we could not isolate protozoa, mycoplasmas, spirochetes, cellulolytic bacteria, pectolytic *Erwinia* spp., or anaerobic spore-forming bacteria. Populations of total bacteria were composed of fluorescent pseudomonas and non-fluorescent bacteria on all leaf pieces. About 90% of the fluorescent pseudomonads were biovars of *P. fluorescens*, with biovar 2 being the most abundant on 85% of the leaf pieces. Eighty-five percent of the non-fluorescent bacteria were Gram (-) and include *Enterobacter* spp., *Rahnella aquatilis, Serratia* spp., *Chryseomonas luteola, Xanthomonas maltophilia, Flavobacterium* spp., *P. paucimobilis* , and *Acinetobacter* spp. based on identifications with API reaction galleries and the Biolog Microstation. Yeast-like organisms were detected on all and filamentous fungi on only 30% of the leaf pieces. Total bacterial populations ranged from 100 to $6x10^8$ cfu/cm^2. Leaf pieces with nascent soft rot symptoms after 7 days storage had total populations of 10^5 to 10^8 cfu/cm^2, whereas pieces with total populations exceeding 10^8 cfu/cm^2 were free of decay. The proportions of yeasts, fluorescent pseudomonads and pectolytic bacteria in the total population were not apparently related to total population size or presence of soft rot symptoms. Preliminary results of inoculations of surface-sterilized scarole leaf pieces indicate that *X.*

maltophilia , *P. paucimobilis* and *F. multivorum* as well as *P. viridiflava* and biovars of *P. fluorescens* can cause decay and discoloration in experimental sachets, but the severity and type of symptoms they cause vary among inoculation trials.

Keywords: *Chicorium endivia*, post-harvest pathology

Ready-to-use (RTU) salads (pre-cut, washed and packaged lettuce or chicory, for example) occupy an expanding market in Europe and the US. In the US the principal RTU salads are composed of cut lettuce (*Lactuca sativa*) where 225000 tonnes were sold in 1987 (King and Bolin, 1989). In contrast, the French market is comprised almost entirely of mixtures of different varieties of *Cichorium endivia* , with a sales volume of 35000 tonnes in 1989 (Varoquaux, personal communication).

RTU salads may discolor or decay during storage, posing important losses particularly in countries where the recommended date of limit of consumption is about one week after fabrication. The physiology of the cut salad tissue as well as its microbial load may influence deterioration during storage (Kader, 1986; Nguyen-the and Prunier, 1989), although the relative importance of these factors has not been established. *Pseudomonas viridiflava* and biovars of *P. fluorescens* have been implicated in decay, but we have observed that decay of RTU scarole (*C. endivia*) may occur in the absence of detectable populations of fluorescent pectolytic bacteria. Pectolytic *Erwinia* spp. are rarely found on RTU scarole (N'guyen-The and Prunier, 1989), and we suspect that organisms which are normally saprophytic may contribute to the decay of this fragile product.

The objective of this work is to identify the microorganisms associated with RTU scarole that promote decay of scarole pieces in sachets. To meet this objective we have attempted to isolate practically all culturable microorganisms associated with commercially available sachets of scarole and to test representatives of each identified species for their ability to produce decay in experimentally prepared sachets. Here we report preliminary results obtained for our bacterial isolates.

MATERIAL AND METHODS.

Isolation of microorganisms: Eight leaf pieces from each of 8 sachets of RTU scarole of different origins were randomly sampled at one or 7 days after fabrication of the sachets and ground individually in 20 ml of sterile phosphate buffer with an ultra-turax. The homogenate was filtered through sterile cheese cloth. Aliquots of the filtrate were i) heated to 70 C for 10 min and dilution plated on nutrient agar, ii) filtered and plated for isolation of spirochetes (Canale-Parola,

1984), iii) filtered, enriched and plated for mycoplasms (Tully, 1984) and *Bdellovibrio* spp. (Burnham and Conti, 1984), iv) enriched and plated for coliforms (Denis and Picoche, 1986), *Salmonella* spp. (Le Minor, 1984), *Shigella* spp. (Rowe and Gross, 1984), *Staphylococcus* spp., *Micrococcus* spp. (Schleifer, 1984), enterococci (Denis and Picoche, 1986) and streptococci (D-group) (Hardie, 1984), v) concentrated on a 0.22 μm filter and observed directly under the microscope for protozoa and vi) dilution plated directly on one of about 20 selective, differential or general media. The media used for direct dilution plating were 10% tryptic soy agar supplemented with cycloheximide (50 mg/L) for isolation of aerobic mesophilic bacteria, acidified potato dextrose agar (Difco) supplemented with streptomycin (200 ppm) for yeasts and filamentous fungi, Paton's medium with cycloheximide for differentiation of pectolytic bacteria (Paton, 1959), cellulose agar with cycloheximide for differentiation of cellulolytic bacteria (Stackebrandt and Keddie, 1986), King's medium B with cycloheximide for differentiation of fluorescent pseudomonads (King *et al*, 1954), glucose yeast extract $CaCO_3$ medium for differentiation of *Frateuria* spp. (Swings *et al*, 1984); D2, D3 and D5 media for *Corynebacterium* spp., *Erwinia* spp. and *Xanthomonas* spp., respectively (Kado and Heskett, 1970); MRS, Rogosa's and Mayeux's media for lactic acid bacteria (Guiraud and Galzy, 1980); Mac Conkey's medium (Difco), violet red bile glucose agar and GLD medium for *Enterobacteriaceae* (Guiraud and Galzy, 1980); KF medium (Difco) for *Streptococcus* spp. (Hardie, 1986), FTO medium for *Micrococcus* spp. (Schleifer, 1986) and CNS medium for *Curtobacterium* spp (Gross and Vidaver, 1979). Media were incubated under aerobic conditions at 20, 22 or 37 C or under microaerophilic or anaerobic conditions at 22 C for 2-12 days. After incubation, each colony on each medium was categorized based on its morphology and representatives of all colony types on all media were purified. On differential media, only those colonies resembling the description of the organisms of interest were sampled.

Bacterial identification: After purification, isolates were tested for production of fluorescent pigment on King's medium B (King *et al*, 1954). Non-fluorescent isolates were tested for Gram reaction (Cerny, 1976), oxygen relations and presence of oxidase (Gerhardt *et al*, 1981). Presence of catalase was determined for Gram (+) isolates. The identity of isolates clearly obligately aerobic or facultatively anaerobic was determined with API 20 non-enteric and API 20 enteric reaction galleries, respectively. Isolates with ambiguous oxygen relations or those that could not be identified with API galleries were characterized with the Biolog microstation and reaction plaques (Bochner, 1989).

Fluorescent isolates were tested for presence of oxidase and arginine dihydrolase (Gerhardt *et al*, 1981). Oxidase (-) isolates were differentiated based on utilization of D(-)tartrate. Oxidase (+)-arginine dihydrolase (+) isolates were differentiated based on production of levan from sucrose, of chlororaphin or pycocyanin on King's medium A (King *et al*, 1954) and of lipase and gelatinase; utilization of L(+)tartrate, D-xylose and D-alanine; and their ability to denitrify NO_3 (Gerhardt *et al*, 1981) and to grow at 41 C.

Inoculations of scarole: Surface-sterilized (10% H_2O_2, 2 min) 5 x 5 cm leaf pieces of chamber- or greenhouse-grown scarole were sprayed (10 ml inoculum / 20 leaf pieces) on both surfaces with a suspension (10^8 cfu/ml sterile 0.05M phosphate buffer) of a 48-hr culture of the bacterial isolate of interest. The surfaces were allowed to dry and each piece was individually sealed in a polypropylene sachet. Sachets were incubated at 4 C and at 10 or 15 C for 7 to 14 days. The percent leaf area decayed was recorded every two days with the aid of a transparent grid. Control treatments included inoculations with sterile buffer and with a strain of *P. fluorescens* biovar 3 known to cause decay under these conditions.

RESULTS AND DISCUSSION.

Total bacterial populations ranged from 100 to 6 x 10^8 colony-forming-units (cfu)/cm^2 and were 10 to 1000 times greater than those of filamentous fungi or yeasts on all leaf pieces examined. Leaf pieces with nascent soft rot symptoms after 7 days storage had total populations of 10^5 to 10^8 cfu/cm^2, whereas pieces with total populations exceeding 10^8 cfu/cm^2 were free of decay. The proportions of yeasts, fluorescent pseudomonads and pectolytic bacteria in the total population were not apparently related to total population size or the presence of soft rot symptoms.

From the leaf pieces sampled, we could not isolate mycoplasmas, spirochetes, cellulolytic bacteria, pectolytic *Erwinia* spp. or anaerobic spore-forming bacteria. Protozoa-like organisms were seen in samples prepared for direct observation but could not be identified or cultured.

Fluorescent pseudomonads composed 1-70% of the total bacterial population on scarole pieces. Fewer than 5% of the fluorescent bacteria isolated were *P. viridiflava, P. syringae* or *P. cichorii.* Most fluorescent bacteria isolated were oxidase (+) - arginine dihydrolase (+); *P. fluorescens* biovars 1, 2, 3, 4 and 5, *P. putida, P. aureofasciens,* and *P. chlororaphis* were detected. *P. fluorescens* biovar 2 was dominant among the fluorescent bacteria on 85% of the leaf pieces.

Among the non-fluorescent bacteria isolated, 85% were Gram (-). Currently, one third of these Gram (-) bacteria have been identified and include *Enterobacter* spp., *Rahnella aquatilis, Serratia* spp., *Chryseomonas luteola, Xanthomonas maltophilia, Flavobacterium* spp., *P. paucimobilis* , and *Acinetobacter* spp. Members of the *Enterobacteriaceae* were predominant as previously reported for RTU scarole (Nguyen-the and Prunier, 1989). About 10 % of the Gram (-) non-fluorescent bacteria isolated were oxidase (+) facultative anaerobes whose presence has not been previously reported on prepared salads of scarole or lettuce.

Table 1. Mean severity (percent leaf area) of laminar discolorations, veinal browning and soft rot on scarole leaf pieces after inoculation with bacteria isolated from ready-to-use scarole and incubation in sachets for 4-5 days at 10 or 15 C. Values preceded by "*" are significantly greater (p < 0.10) than those of the buffer-inoculated control based on analysis of variance.

Symptom:	Laminar discolor.[1]			Vein browning			Soft rot		
Inoculation trial[2]:	1	2	3	1	2	3	1	2	3
Inoculum									
Pseudomonas viridiflava - 021		18.9			*5.5			0.0	
P. viridiflava - 152		2.4			0.4			0.0	
P.. fluorescens biovar 1 - 155		*27.7			0.0			0.0	
P. fluorescens biovar 2 - 007		8.6			*3.0			0.0	
P. fluorescens biov ar 2 - 034			*29.5			*37.5			0.0
P. fluorescens biovar 2 - 079			14.6			0.0			0.0
Xanthomonas maltophilia - 022	0.0	2.9	27.0	*4.6	0.8	0.0	*5.1	0.0	0.0
X. maltophilia - 036			27.0			*37.5			0.0
Flavobacterium sp. - 152	*15.6		4.4	0		1.6	0.0		0.0
Flavobacterium sp. - 156		0.0	*32.7		0.0	0.0		0.0	0.0
Enterobacter agglomerans - 006	8.0			1.3			0.0		
E. agglomerans - 050		9.9			0.0			0.0	
P. paucimobilis - 013	0.0			0.8			1.7		
P. paucimobilis - 015		12.0			*4.8			0.0	
P. paucimobilis - 048	0.0			0.0			0.0		
P. putida - 163			4.5			0.0			0.0
P. putida - 304			9.8			0.0			0.0
Rahnella aquatilis - 001	0.0		5.6	1.0		0.0	0.0		0.0
R. aquatilis - 175			3.2			0.0			0.0
Controls:									
P. fluorescens biovar 3 - T53	0.0	*36.6	*31.5	0.0	0.0	*75.0	*87.6	0.0	0.0
buffer	0.0	5.0	14.8	0.4	0.0	0.1	0.0	0.0	0.0

[1] In trials 1 and 2, laminar discolorations often appeared as a generalized purple or brown; in trial 3 only marginal necrosis was observed.

[2] For trials 1 and 2, ten leaf pieces were inoculated for each isolate or control and incubated at 15 C. For trial 3, eight pieces per treatment were inoculated and incubated at 10 C.

The strains tested to date for their ability to provoke alterations of scarole in sachets are presented in Table 1. At 4 C, leaf pieces in all treatments remained free of decay or discolorations for up to 5 days except for those inoculated with the *P. fluorescens* biovar 3 control strain. At 10 and 15 C strains of *P. viridiflava, P. fluorescens, P. paucimobilis, X. maltophilia* and *Flavobacterium* sp. caused discolorations of the leaf pieces, but no strains caused soft rot before 5 days (Table 1). After 7 days at 10 or 15 C buffer-sprayed leaf pieces as well as pieces inoculated with bacteria were often decayed or discolored.

The type of symptom observed for a given isolate varied among the different trials (Table 1). In particular, greenhouse-grown plants seemed to react to inoculation differently than chamber-grown plants. Likewise, differences in response to inoculation were observed between older and younger leaves. Currently, we are trying to determine which factors of host physiology may be important for bacterial-induced alterations of RTU scarole.

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Bacterial spot diseases of peas, lupine and horse beans in Lithuania

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ABSTRACT

Bacteria were isolated from peas (*Pisum sativum*), lupine (*Lupinus luteus*) and horse beans (*Vicia faba*) infected by spots on the territory of Lithuania in 1982-1988. On the basis of a comparative study of morphological, biochemical, pathogenic and serological properties, (of these bacteria), they were ascribed to *Pseudomonas syringae* group.

KEYWORDS

Pseudomonas syringae pv. *pisi*, *P. lupini*, *P. syringae* pv. *syringae*, peas, lupine, horse beans, agent identification.

INTRODUCTION

In Lithuania detailed studies on bacterial diseases of leguminous plants (spot diseases included) started only in 1970 (PUIPIENE & VASINAUSKIENE, 1988). However, investigations on bacterial diseases of horse beans have not been carried out at all.

Leguminous plants play an important role in the agriculture of Lithuania. Therefore, detailed investigations on bacterial diseases of peas, lupine and horse beans have been carried out in our Republic. The purpose of these investigations was to isolate bacterial agents from the affected plants and to study their biological properties in order to identify isolates.

MATERIALS AND METHODS

The observations on bacterial diseases have been carried out in the main regions of peas, lupine and horse beans cultivation in Lithuania.

The studies on biological properties of isolated strains were carried out by means of general methods (BELTIUKOVA *et al.*, 1968 ; LELLIOT & STEAD, 1987). The biochemical and other properties of isolated strains were studied comparatively with the data obtained in the literature of the subject

(OVETCHNIKOVA, 1971 ; KRIEG & HOLT, 1984 ; KLEINHEMPEL *et al.*, 1989). The serological studies were used for a more complete and detailed identification of bacteria and for diagnostic relationship of investigated strains with the bacteria of *Pseudomonas syringae* group. We employed 12 serums obtained at the Institute of Microbiology and Virology of the Ukraine for this purpose. Serological tests were carried out according to the scheme worked out by PASTUSHENKO and SIMONOVITCH (1979).

RESULTS AND DISCUSSION

During the microbiological analysis of peas, lupine and horse beans affected samples, the pathogenic strains of bacteria were isolated from necrotic spots on the different vegetative parts of plants. Agents were isolated in the second half of summer in phases of flowering and pod formation.

The spots phases in these early stages of development were of different size, rather small, had a water-soaked appearance, dark green in colour, later they increased, got drier and became brown on peas and lupine, while on horse beans they were black. When the plant was heavily affected it looked like burned and that is characteristic when plants are affected by bacteria of *P. syringae* group. During our observations symptoms of diseased plants were identical to those described in literature (YOUNG & DYE, 1970 ; BELTIUKOVA & KOROLIOVA, 1971 ; GRIESBACH, 1976).

The climatic and microbiological conditions are favourable for bacterial diseases of peas, lupine and horse beans in Lithuania. Besides that a more sensitive development of their diseases was observed in years with prolonged rains and higher average monthly temperature (more than 18-20°C). During such years the amount of peas infected by bacterial blight (*P. syringae* pv. *pisi*) made up 30% in the observation regions in 1983, and necrotic areas on their surface made up from 2 to 5%. In 1987 lupine bacterial blotch (*P. lupine*) and in 1984 horse beans blight (*P. syringae* pv. *syringae*) infected 40% of plants. In other years infection was insignificant and was observed only on single plants. Necrotic areas on their surface covered 4-5%.

Strains isolated from peas, lupine and horse beans were similar to bacteria of genus *Pseudomonas* according to their cultural and morphological properties. Colonies on potato agar medium were grey, round with elevated centre and uneven edge. The cells were Gram-negative thin long motile rods.

Pathogenicity of the isolates was tested by inoculation of host plants. Bacteria indicated positive reaction on tobacco leaves and they did not grow on

potato slice. The principal biochemical properties of strains did not differ from each other and complied with the data described in Bergey's Manual (KRIEG & HOLT, 1984). Certain differences were registered only in utilization of carbon sources (Table).

Table : **Comparative data on biochemical properties of pathovars** *Pseudomonas syringae*

Test	*P. syringae* pv. *pisi*		*P. lupini*		*P. syringae* pv. *syringae*		*P. syringae* pathovars
	isolated	Kleinhempel *et al.*, 1989	isolated	Beltiukova & Koroliova 1971	Isolated	Ovetchnikova 1970	Bergey's Man., 1984
1	2	3	4	5	6	7	8
Denitrification	-		-		-		-
Levan formation from sucrose	+	+	+		+		+
Nitrate reduction	-	-	-	±	-		-
Lecithinase (egg yolk)	-		-		-		d
Oxidase	-	-	-		-		-
Catalase	+	+	+		+		+
Gelatin lique-faction	+	+	+	+	+		d
Utilization of :							
arabinose	+	+	+	+	+	+	d
xylose	±	+	+	+	+		d
rhamnose	-		-	+	-		d
glucose (aerobically)	+	+	+	+	+	+	+
glucose (unaerobically)	-		-		-		
fructose	±	±	+	+	+		d
galactose	±	±	+	+	+	+	d
lactose	-	-	-	-	-	-	-
sucrose	±	+	+	+	+	+	d
maltose	-	-	-	-	+		-
raffinose	±		-	+	-	-	d
mannitol	-		±	+	-	+	d

Note : + presence of property ; - absence of property ; ± variable reaction ; d 11-89% of strains are positive ; empty space : absence of data

Studies on biological properties of agents of lupine bacterial blotch, parasitizing in Lithuania, and their comparison to the data in literature (BELTIUKOVA & KOROLIOVA, 1971) indicated a high level of identity with the bacteria isolated in the Ukraine and which were called *Pseudomonas lupine* Beltiukova-Koroliova. The particular differences were registered in nitrate reduction and utilization of some carbon sources which might occur due to strain isolated under different geographical conditions. However, the principal properties of isolated strains according to LOPAT tests (LELLIOTT *et al.*, 1966) indicated close identity to pathovars *P. syringae* group in Bergey's Manual (KRIEG & HOLT, 1984).

Serological reactions of agglutination allowed to determine common antigenes in isolated strains and collection strains of *P. syringae* group. The isolated strains from peas agglutinated in high dilutions with serum to *P. syringae* pv. *pisi* culture, from lupine - to *P. lupine*, from horse beans - to *P. syringae* pv. *syringae*, and that reveals their close identity to these species.

Thus, in the territory of Lithuania bacterial spot-injured peas, lupine and horse beans were determined and their agents isolated. A comparison of morphological, biochemical, pathogenic and serological properties of these isolates with the properties of agents described in the literature (KRIEG & HOLT, 1984) enabled to ascribe them to pathovars of *Pseudomonas syringae* group. It was determined that peas are infected by *Pseudomonas syringae* pv. *pisi* (SACKETT, 1916 ; YOUNG *et al.*, 1978), lupine - by bacteria identical to *Pseudomonas lupini* Beltiukova-Koroliova, horse beans -*Pseudomonas syringae* pv. *syringae* VAN HALL (1902).

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Serological and molecular reagents for the detection and characterization of the bacterium-like organism (BLO) of citrus greening disease

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Greening is one of the most serious diseases of citrus. It is caused by a phloem-restricted, non-cultured, bacterium-like organism (BLO) [1]. In 1987 we have produced monoclonal antibodies (MAs) specific for the greening BLO by using phloem purified from BLO-infected plants as the immunogenic preparation [2]. The greening infected material that was used to produce MAs was from Poona (India). With these MAs it was easy to detect the greening BLO in infected plants by ELISA or immunofluorescence. However, the MAs were highly specific for the Poona BLO and did not recognize BLOs from other geographical origins. MAs produced against BLOs from China and South Africa have confirmed these results [3]. This shows that different serotypes of the greening BLO occur and that MAs are too specific for diagnostic purposes. We have now produced DNA probes that can recognize all BLO strains tested [4]. Their use for the detection of the BLO in plants and insects will be illustrated. Sequencing of the probes has revealed that one of them contains the rplKAJL-rpoBC gene cluster with the same organization than in eubacteria [5].

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Monoclonal antibodies and DNA probes for the study of MLOs in plants and insects

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Monoclonal antibodies (MAs) specific for the MLOs of tomato stolbur and witches' broom disease of lime trees (WBDL) in Oman have been produced [1,2]. DNA probes were also obtained for the WBDL MLO [2]. Using these reagents we were able to detect the respective MLOs in plants and insects. This has allowed us to identify *Hyalesthes obsoletus* as the insect vector of the stolbur MLO in tomato and other plants in south western France [3].

Only one leafhopper species, *Hishimonus phycitis*, was found to be infected with the WBDL-MLO in Oman. Experimental transmissions of the MLO to healthy lime seedlings are underway to demonstrate that *H. phycitis* is the vector of the disease.

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Use of polyclonal antibodies to identify mycoplasmalike organisms (MLOs) from the Sudan and from Thailand

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ABSTRACT

Rabbit polyclonal antibodies prepared in Versailles against faba bean phyllody MLO from the Sudan reacted with homologous antigen and with extracts of *Catharanthus roseus* experimentally infected with the same or a related MLO from *Crotalaria saltiana* showing symptoms of phyllody disease, as well as with extracts of naturally MLO infected *C. saltiana* growing in the field in the Sudan. The antibodies also reacted positively with extracts of *C. roseus* experimentally infected with *Crotalaria juncea* phyllody MLO or soybean phyllody MLO from Thailand.

Polyclonal antibodies prepared at East Malling against an MLO associated with a witches' broom disease in *C. juncea* from Thailand reacted positively in ELISA tests with homologous antigen extracts from infected *C. juncea* as well as with extracts from experimentally infected *C. roseus* and with extracts prepared from *Sesamum indicum* with phyllody symptoms growing in Thailand. There was no reaction between these antibodies and extracts from *C. roseus* plants infected with the MLOs associated with *C. juncea* phyllody or with soybean phyllody.

No cross reactions were observed among the antigens and antibodies of the two MLO groups by ELISA or western blot. However, the molecular weight of the principal protein antigen, determined following SDS polyacrylamide electrophoresis (SDS-PAGE) and western blotting was the same for both types of MLO.

We conclude that serologically similar MLOs occur in the Sudan and in Thailand, where they are associated with phyllody symptoms in *C. saltiana* and faba bean and with *C. juncea* and soybean, respectively. We suggest that soybean crops growing in the Sudan should also be examined for the possible occurrence of this MLO. A second, serologically distinct MLO group was also found infecting *C. juncea* and *S. indicum* in Thailand but MLOs from this group have not yet been identified in crops from the Sudan.

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Bacterial leaf spot and stem rot disease of carnations

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A serious leaf spot and stem rot disease of carnations, caused by the bacterium *Pseudomonas andropogonis*, was responsible for two epidemics in Australia during 1987 and 1988. Epidemiology and control of the disease were studied in the glasshouse. Disease development was found to be largely dependent on temperature and leaf wetness. The optimum temperature range for symptom development is 23-27°C, whilst a temperature of 15°C retards development.

Over 50 cultivars of carnations from three leading propagators were screened for resistance to the disease. Plants were assessed over four separate trials by rating them according to the percentage of leaf area affected by the disease. Complete resistance to the bacterium was detected in some of the cultivars of the "Diana" and "Micro" groups; "Sprays", "Fields", "Sims" and "Standards" showed a range of susceptibility to the disease. The type of resistance observed in carnations had characteristics of polygenic or horizontal resistance. The efficacy of chemicals to control the disease was evaluated over three trials. Forty treatments were tested which consisted of various chemicals at different rates and in a variety of combinations. There were no chemicals or combinations of chemicals, which completely eliminated the disease, though some chemicals reduced its severity. Control of the disease in Victoria, Australia will have to rely on growing resistant cultivars especially when plants are grown outside. The alternative methods, adopted by most growers, involves growing plants under cover and avoiding overhead water. In propagating beds it has been controlled by dipping cuttings in sodium hypochlorite and with good hygiene practices.

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Black spot of lamb's lettuce, a new bacterial disease

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The production of lamb's lettuce, *Valerianella Locusta*, is traditional in France in Nantes area. In the past, the normal season of harvest was Autumn and Winter but from some years practical techniques evolve to grow lamb's lettuce continuously. In this new climatic conditions, the pathogenic complex had changed. The important diseases, *Peronospora valerianellae* and *Phoma valerianellae* dicrease with good chemical control while news symptoms appear.

Under moist climatic conditions, frequently just before the harvest, watersoaked spots appear on the leaves. After 3 or 4 days they become black. They are circular with a regular margin. The size can be reach 2 or 3 mn in diameter. But generally the first symptoms appear also on the cotyledons just after emergence of the young plants. When the number of spots is too important, lamb's lettuce will not harvest because the production is unmarketable.

From this symptoms a bacteria is regurlarly isolated. The colonies appear slowly on LPGA medium. They are never mucous. It is a gram-, strictly aerobic bacillus with one polar flagella. On King B medium, any fluorescent pigment is produced. Oxydase and urease are positive but arginine and levane are negative. Nitrates are not reduced.
ßpolyhydroxybutyrate is not accumulated. Just after isolation, a high concentration of bacteria give hypesensitive reaction on tobacco leaves. On the basis of these characters we assigned this bacteria closed to *Pseudomonas*. This bacteria does not correspond with any previously described phytopathogenic *Pseudomonas*.

We reproduced regularly all typical symptoms by artificial inoculation. Moreover, the symptoms appear on the cotyledons after bacteria inoculation of soil or also seeds just before sowing.

Complementary studies are going to specify the taxonomic position of this bacteria.

III

New approaches in taxonomy Identification and phyllogeny

Plant Pathogenic Bacteria, Versailles (France), June 9-12, 1992
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Changing concepts in taxonomy

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The paper delivered at the conference was a resumé of Young *et al.* (1992) and need not be further abstracted here. These notes present an elaboration of points made in Young *et al.* (1992), and further developed from discussions at the conference.

Sequence Evolution and Molecular Clocks

rRNA sequence analyses are the basis of many bacterial phylogenetic reconstructions on the assumption that expressed mutational changes occur in a clock-like manner (Woese 1987). However, it is also accepted that the structure of rRNA and all other conserved sequences is closely regulated by natural selection to optimize its function in protein synthesis. The extent to which rRNA is conserved in accord with functional requirements is clearly illustrated when sequence similarities and differences between genera are compared. rRNA of species within a genus is largely made up of identical sequences, distinct from those of other closely related genera. Only intense selective pressure for effective function on genus-and species-specific sequences operating in the cellular environments of related organisms, during their derivation from a common ancestor, can account for these regularities. It is these same sequences which are considered to change in a constant way with time (Woese 1987). For this to occur, it is necessary to assume that selective changes occur at a constant rate in shared homologous (sharing a common ancestry) sequences in unrelated organisms. However, for other homologous sequences, change must occur at different but constant expressed rates, also imposed by natural selection. As yet, detailed comparisons by sequence

analysis have not rigorously confirmed the assumptions of these models for bacteria. So far, the probability or otherwise of this model of evolution has not been questioned.

Many classifications, particularly for higher organisms, have been reported in which sequence analyses do conform to clock-like behaviour. However, other analyses based on apparently equivalent data using similar methods of analyses do not have this character and can produce classifications which are contrary to common sense and the evidence of the fossil record. It is possible that many of these go unreported. The acceptance of classifications based on molecular clocks will be widespread when more comprehensive studies show that consensus states can be achieved from different sequences. A proper scepticism would follow if it transpired that classifications conforming to plausible molecular clocks represented a small proportion of the families of classifications produced by sequence analyses, and if there were no rational procedure for determining the validity of one classification over another.

Many different algorithms have been applied to produce phylogenetic reconstructions (Woese 1987). A distinction needs to be drawn between models based on clustering algorithms, which produce phenetic relationships, and those based on tree-building algorithms which, with appropriate assumptions, may indicate ancestral relationships. For the latter, a second distinction needs to be drawn between algorithms that produce trees which are characterized by order of branching and those in which both branch order and length of branches are components of the pattern. Although branch order may plausibly reflect phylogenetic orders of divergence, branch order alone does not determine uniquely which taxa at any level should be grouped in a higher taxon. Thus, from branch orders for n taxa, it is possible to produce $(2n-4)!/2^{n-2}(n-2)!$ equivalent classifications. Only by the consideration of phenotypic data, or the introduction of additional data which take account of branch length differentials, is it possible to produce unique classifications.

Parsimony and Phylogenetic Reconstruction

The principle of parsimony is a derivation of Occam's axiom which, applied to scientific theories, states that these should be no more complicated than is

necessary to account for relevant observations. Parsimony, applied to phylogenetic reconstruction, involves assumptions which minimize the complexity of branch patterns. At the molecular level it assumes that mutations in sequences occur irreversibly and that the evolutionary path connecting two sequences will be that which involves the minimum number of implied mutations. However useful the theory may be for evaluating scientific theories, its utility in resolving genetic histories is less self-evident (Friday 1982). Extant Baroque molecular regulatory mechanisms (such as the tryptophan operon) highlight the potential complexity of evolutionary history exemplified by divergence, stasis, and parallelism. The simple evolutionary pathways implied by analyses assuming parsimony may conceal a much more complex evolutionary history (Doolittle 1988). Generating phylogenetic trees can be considered as analogous to hypothesizing the actual branch structures of a living tree by an analysis of the relative positions of its leaves. For some trees, a parsimonious analysis (or analysis based on any other assumptions) from the leaf arrangement may lead to the derivation of a model branch pattern which corresponds to the actual structure. For others, the model may vary considerably from reality. Clearly, internal evidence supporting a particular phylogenetic reconstruction needs to be considered rigorously in relation to its competitors before any particular pattern is accepted even as an approximation to reality.

Gene Transfer or Independent Evolution?

Phylogenetic reconstructions depend on the assumptions that significant gene transfer does not occur between taxa and that identical genes do not evolve independently to an extent that could cause taxonomic confusion. If these assumptions are not made then organisms could evolve as composites of transferred genes (Doolittle 1987) and taxa could be the product of unrecognized convergent evolution. However, there are instances where either gene transfer or independent evolution is indicated in secondary cell functions. For example, syringotoxin is produced in some strains of *P. syringae* and in *P. fuscovaginae* (Pelsser *et al.* 1992). Identical toxins are found in genomically distinct pathovars of *P. syringae* (Mitchell 1991). The genes for the toxins of xanthomonads pathogenic to *Cassava* spp. (*Xanthomonas campestris* pv. *cassavae* and *X.c. manihotis*) appear to have evolved independently in geographically distinct populations. By their

nature, instances of these phenomena will be hard to distinguish from convergent evolution or even to identify except as anomalies, if they occur in central cellular functions.

Evolution, Saprophytes, Avirulence and Nomenclature

Plant pathogenic bacteria evolved from pre-existing free-living bacteria, probably saprophytic forms. Most pathogenic taxa (*P. solanacearum, P. cepacia* and the soft-rotting pseudomonads may be exceptions) have become nutritionally fastidious and may have lost the capacity for survival in environments where they are in constant competition with free-living organisms. Most studies have confirmed the progressive decline of pathogenic populations in the absence of their supporting host. However, reports have been made of strains identified in *Xanthomonas* (Stall & Minsavage 1989, D.E. Stead, pers. comm.) and *P. syringae* (Hirano & Upper 1990), isolated from plant associations, but for which pathogenicity to any host has not been proved. As well, instances have been recorded of pathogens surviving in soil as free agents (Goto, 1972). It is still a matter for speculation whether these strains represent populations pathogens living as true saprophytes, capable of existence independently of any living plant, or if they are casual survivors from a pathogenic interaction. If they are from a pathogenic population which does not survive independently of a plant interaction, then they could either be virulent strains which have not succumbed in saprophytic competition, virulent strains pathogenic to an unidentified host, or could be avirulent mutants, representative of moribund sub-populations. If they represent pathogens which also have a true saprophytic phase, then this might be reflected in a non-fastidious nutritional capability and the organisms will be found at further remove in time and space from living plants.

An area of study which will illuminate both the nomenclatural and pathogenic concepts affected by this question involves the investigation of weak pathogens and the role of latent infections (Hayward 1974) in the seasonal cycle of pathogenic bacteria, as well as detailed studies of pathogenic bacteria from mixed populations made possible by nucleic acid probes (Stackebrandt & Goodfellow 1991). Hirano & Upper (1989) suggested that if data from saprophytic strains of *P. syringae* had formed the basis of the species description, then nomenclatural confusion would have been avoided. Had this been the case, then the nomenclature of *P. syringae*

would be analogous to that of *Erwinia herbicola* or any other saprophytic species for which pathogenic variants are subsequently described (Young *et al.* 1992). There need be no nomenclatural uncertainty provided that species names are not confused as identifiers of pathogenic strains as such. Thus, although species names are often the designations of causal agents of specific diseases, names such as *P. syringae* or *Erwinia amylovora* of themselves should not be taken to imply the pathogenicity of specific strains.

The Reinstatement of Genomic Species

DNA-DNA hybridization of representative strains of *Xanthomonas* shows that *X. campestris* can be sub-divided into at least twelve genomic species (Vauterin *et al.* 1992). On this basis, a large number of pathovars would be allocated to *X. axonopodis*. Other pathovars would be allocated to appropriate genomic species. This genomic analysis is supported by protein profile data (Swings, pers. comm.) but not by fatty acid profile data (Vauterin *et al.* 1992). There is no data for determinative keys for these species. If they are formally reinstated, then for practical purposes, it will only be possible to infer species identifications if pathogenicity data is available. This should not lead to confusion for diagnosticians if the principles of application are explained when these names are promulgated. However, for systematists, the implications of this step should not be lost sight of; the species rank will now have the taxonomic characteristics previously associated with the infrasubspecific rank. This kind of classification can only be applied indirectly by making identifications using data other than that obtained by examinations of isolates. If generally applied, such classifications will ultimately fail to fulfil their taxonomic function unless alternative direct identification procedures are developed (Young *et al.* 1992).

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Identification of Gram negative plant pathogenic bacteria by the biolog microplate system for carbohydrate utilization

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ABSTRACT

Approximately nine hundred *Agrobacterium, Erwinia, Pseudomonas and Xanthomonas* strains were tested for carbohydrate utilization by adding a bacterial suspension to each well of GN MicroPlates (Biolog, Inc., Hayward, CA) with each of 95 wells containing a different carbon source (amino acids, carboxylic acids and sugars). Reduction of a tetrazolium dye resulted in the formation of a purple colour. The resulting reaction pattems were read with a microplate reader and processed with the MicroLogTM database to identify to genus, species and where applicable pathovar. The system identified over 95% of the strains to correct species. Cluster analysis revealed that *X. campestris* and *P. syringae* groups formed fairly tight clusters indicating difficulties in correctly identifying pathovars. Many of the relationships observed between bacterial species in this study were also observed in other studies.

INTRODUCTION

Standard methods for the identification of phytopathogenic bacteria to genus and species in diagnostic laboratories rely on biochemical and physiological tests (SCHAAD, 1988). Although these tests are useful, they require considerable time and expense. Information based on phenotypic characteristics such as carbon utilization patterns can provide much of the needed information for identification of a bacterium to genus, species or even sub species level. However, the factor of time precludes using utilization patterns of numerous carbon sources for routine identification. API strips (API System, Montalieu, France) have been invaluable for clinical laboratory diagnosis by creating an easier system for performing carbon utilization patterns and also standardizing the process.

HAYWARD *et al.* (1989) differentiated biovars of *Pseudomonas solanacearum* by running a range of biochemical and physiological tests including carbon source utilization and acid production of a range of carbohydrates. The use of microtiter plates and a plate reader offers the ability to enter the data into a computer. BOCHNER (1989) has developed a microplate system (Biolog, Inc., Hayward, CA, USA) for identification of gram negative bacteria. This system is based on the reaction patterns to a series of carbon sources with positive reactions revealed by the reduction of tetrazolium violet to a purple formazan. The resulting reaction pattern is called the bacterium's "metabolic fingerprint". Interfacing the microplate system with a database by direct entry of the reaction pattern into the computer and identification by determining the percent similarity with reaction patterns in the database allows rapid identification. This paper reports on the use of the microplate system by Biolog for identification of Gram negative plant pathogenic bacteria.

MATERIALS AND METHODS

Numerous (915) strains comprising species of *Agrobacterium*, *Erwinia*, *Pseudomonas* and *Xanthomonas* were grown on Trypticase Soy Agar (TSA from BBL) for 18 h at 28°C. Cells were suspended in sterile saline and adjusted to 0.17 to 0.19 optical density at 590 nm. Each well of the GN MicroPlates^{TM} was inoculated with 150 µl of the bacterial suspension. Plates were incubated 4 and 24 h at 28°C before being read with a plate reader (EAR400 AT SLT-Labinstruments, Austria). Results were analyzed with the MicroLog^{TM} database (Release 2.01) to determine the identity of each strain and a new database was made consisting of these strains and those already present in the original MicroLog^{TM} database. The reaction patterns were then analyzed a second time to determine if identification was improved.

RESULTS AND DISCUSSION

With most of the strains tested, the 4 h reading was generally less informative compared to the 24 h readings. With 603 samples run on 525 strains from 25 different pathovars of *Xanthomonas campestris*, 100% of the strains were correctly identified to species whereas 2% were correctly identified to pathovar.

The initial database was insufficient for identifying many of the strains to

Table 1. Examples of bacterial species tested with the Biolog GN System and the percentage of strains of each species which was identified correctly by the original Biolog™ data base and the updated version.

Organism	Strains	Biolog Database	
		Original	Updated
Erwinia carotovora	23	52[a]	96
Pseudomonas andropogonis	31	96	100
P. cichorii	52	94	100
P. syringae pv. *syringae*	32	0	61
P. syringae pv. *tomato*	15	0	100
Xanthomonas campestris pv. *campestris*	37	3	57
X. campestris pv. *vesicatoria*	28	11	57

[a] Value represents the percentage of strains which were identified correctly to species or pathovar by the original and updated versions of the biolog system.

species and/or pathovar. The lack of precision on identification to pathovar is consistent with the findings of Hartung and Civerolo (1991). Once the database was updated with entries from this study, identification to species and pathovar was improved (Table 1). Several species such as *P. andropogonis* and *P. cichorii* were not affected significantly by updating the database. Although the addition of more *X. campestris* strains from various pathovars to the database improved identification to pathovar, it does not appear that many of the pathovars within *X. campestris* can be identified with a high degree of accuracy to the correct pathovar. Approximately 50% of the strains of *X. campestris* pv. *campestris* and *X. campestris* pv. *vesicatoria* were identified correctly to pathovar. Dye (1962) was not able to discern between 57 *Xanthomonas* nomenspecies by phenotypic characteristics. Similarly, within *P. syringae* many of the strains were not identified correctly to pathovar using the MicroLog™ data base (Release 2.01). The inability to differentiate between many of the pathovars using the biolog system is not contrary to previous studies in which *P. syringae* nomenspecies were not effectively differentiated by various phenotypic tests (Hildebrand *et al.*, 1987; Misaghi and Grogan, 1969; Sands *et al.*, 1971). With *Agrobacterium*, 56

pathogenic and nonpathogenic strains from diverse geographic and host origins segregated according to chromosomal groups (i.e., biovars). However, two additional biovar 3 strains formed a distinct cluster.

Cluster analysis was performed on all strains analyzed in this study and revealed relationships consistent with previous studies (De Ley, 1978; De Smedt and De Ley, 1977; De Vos and De Ley, 1983). The xanthomonads were a fairly closely related group; however, certain xanthomonads were unique compared to most of the others. *Xanthomonas maltophilia* was distantly related to all other members of the genus with the exception of two xanthomonads isolated from *Xanthosoma* and *Syngonium*. Strains from these latter two plant species appear to be distantly related to many of the X. *campestris* pathovars (Chase *et al* 1992). *Xanthomonas maltophila* has also been reported to be distinct from the other xanthomonads and it was proposed that this bacterium be placed in a new genus but be kept in the same general group with the xanthomonads (van Zyl and Steyn, 1992).

The *P. syringae* group was also a very tight group forming a cluster distinct from many of the fluorescent and all nonfluorescent pseudomonads and from the other phytopathogenic bacteria studied. van Zyl and Steyn (1990) demonstrated a similar relationship by analyzing protein gel electrophoregrams of a number of *P. syringae* pathovars. Many more strains need to be analyzed for proper judgment of the Biolog system with regard to accuracy of identification of pathovars within the *P. syringae* group.

The Erwinia group was distantly related to other genera of bacteria with the exception of *Agrobacterium*. *Erwinia amylovora* formed a group isolated from all other *Erwinia* spp. *Erwinia carotovora* and *E. chrysanthemi* formed a close group whereas *E. herbicola* apparently was more closely related to *Agrobacterium* than to the other erwinias. These results are in agreement with previous studies on the relative relationships of the erwinias (Dye, 1968; Dye, 1969a; Dye, 1969b; Dye, 1969c). The *Agrobacterium* strains were distantly related to all other plant pathogenic bacteria studied confirming the work of De Smedt and De Ley (1978).

The Biolog MicroPlate system is a useful tool for characterization of strains. This system can be used as a tool for rapid identification of unknown bacterial

species and for studying relationships between bacterial strains. Addition of more strains to the data base or creation of personal data bases by potential users should improve the accuracy of this system.

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Synonymy of *Pseudomonas syringae* pv. *maculicola* and *Pseudomonas syringae* pv. *tomato*

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ABSTRACT

Bacterial strains of *Pseudomonas syringae* pv. *maculicola* (Mc CULLOCH, 1911 ; YOUNG, DYE & WILKIE, 1978) and *P. syringae* pv. *tomato* (OKABE, 1933 ; YOUNG, DYE & WILKIE, 1978) were compared using physiological, biochemical and pathological tests. When inoculated by spraying, all the strains were pathogenic on both tomato (*Lycopersicon esculentum*) and cauliflower (*Brassica oleracea* var. *botrytis*), producing typical bacterial sped and bacterial leaf spot symptoms, respectively. They were divided into four groups based on several physiological and biochemical tests. However, this grouping did not distinguish the two pathovars. In addition, the pathotype strains of the two pathovars showed the same reactions on these tests. We were unable to differentiate the strains of pv. *maculicola* from those of pv. *tomato*. We propose that they should be treated as a single pathovar, retaining the name *Pseudomonas syringae* pv. *maculicola*.

INTRODUCTION

Pseudomonas syringae pv. *maculicola* (Mc CULLOCH, 1911 ; YOUNG, DYE & WILKIE, 1978) and *P. syringae* pv. *tomato* (OKABE, 1933 ; YOUNG, DYE & WILKIE, 1978) are the causative agents of the bacterial leaf spot of cruciferous plants and bacterial spec of tomato, respectively. They have world-wide distributions and are causing serious damages to growers. We have studied the causal bacterium of bacterial spot of various cruciferous plant in Japan for a decade and have found that the bacterium, identified as *P. s. maculicola*, causes pathogenic reactions both on cruciferous plants and on tomato. Therefore, we compared the Japanese bacterium with the authentic strains of pv. *maculicola* and pv. *tomato*. It was shown that the both pathovars, together with Japanese strains, appeared to be indistinguishable in pathogenic traits.

This paper reports the results of a comparative study of phenotypic properties and pathogenicity of the pseudomonad isolated in Japan and known strains of pv. *maculicola* and pv. *tomato*.

MATERIALS AND METHODS

Total 173 bacterial strains isolated from cruciferous plants and tomato were investigated. Among them, 5 strains of pv. *maculicola* and 6 strains of pv. *tomato* were obtained from ICMP, having various geographic origins. The pathotype strains of the both pathovars were included.

Morphological, cultural, physiological and biochemical properties were investigated according to the methods described previously (TAKIKAWA *et al.*, 1989). Utilization of organic compounds as sole sources of carbon was determined on the medium of AYERS *et al.* (Manual of Microbiological Methods, 1957) solidified with 1.5% agar, incorporating carbohydrates at 0.5% and other compounds at 0.1%.

Inoculations were made on tomato (*Lycopersicon esculentum*), cauliflower (*Brassica oleracea* var. *botrytis*), radish (*Raphanus sativus*), turnip (*Brassica rapa*) and cucumber (*Cucumis sativus*). The seedlings of the plants were inoculated by spraying ca. 10^7 cells/ml of bacterial suspensions without wounding. Coronatine-like activity was determined by the bioassay on potato tuber slices (NISHIYAMA & EZUKA, 1978).

RESULTS

All the strains investigated were Gram negative, aerobic and motile with several polar flagella. They produced fluorescent pigment and formed white colonies. LOPAT tests were + - - - +. They utilized D-mannitol, inositol, trigonelline, D-tartrate and so on, but did not erythritol, L-tartrate or lactate. As for 37 strains selected, nearly 100 phenotypic characters were investigated, and the results were subjected to cluster analysis using simple matching coefficient and nearest neighbour linkage or average linkage methods. Four clusters were identified from the dendograms obtained. Characters which vary among the four groups were listed on table 1. Both pathotype strains of pv. *maculicola* and of pv. *tomato* were assigned to group I, showing the same reactions on these tests with the exception of utilization of L-histidine (ICMP 3935 + and ICMP 2844 -). In addition, these four groups were distinguished by their colony morphology and maximum growth temperature. The groups I and II formed flat, translucent and butyrous colonies, while the groups III and IV formed domed, opaque and viscous colonies. The maximum temperatures for growth of the groups I, II, III, IV were 32-33, 29, 32 and 34-36°C, respectively. The correlation between the host of origin and the group designation of each strains were listed in table 2.

Table 1

Characters in which four groups of pv. *maculicola* and pv. *tomato* complex gave different results

	group I	group II	group III	group IV
	(12)	(10)	(10)	(5)
Purple milk reaction	KD (9)a) A (3)	KD	A	(KD)
Hydrolysis of				
Gelatin	+ (10)	+	-	(+)
Casein	+	+	-	+
Tween 80	+	+ (9)	-	+
Esculin	+	+	±	-
Tyrosinase	-	-	-	+
Growth at 31°C	+	-	+	+
Utilization of				
m-Tatrate	+	- m	+	-
Malonate	+	-	+	-
Leucine	+ (11)	-	+	-
Raffinose	+ (9)	- (1)	-	-
Xylose	+	+	-	+
Sorbitol	+	+	+	-
Caprate	+	+	+	-
Acetate	+	+	+	-
Glutarate	+	+	+	-
Glycerate	+	+	+	-
Tyrosine	+ (11)	+	+	-
Prospionate	V (5)	+ (9)	+	V (2)
Histidine	V (6)	+	+	V (1)

a) symbols used :

K : alkali formation ; D : digestion ; A : acid formation ;

+ : 80% or more strains positive ; V : 20 to 80% strains positive ;

- : 0 to 20% strains positive.

numbers in parentheses show the number of positive strains

Table 2

Relationship between original host plant and group designation of strains

Host plant	number of strains designated to each group			
	group I	group II	group III	group IV
Tomato	8	1	0	0
Cauliflower	4	5	7	0
Radish leaf	0	54	0	23
Radish root	0	38	0	0
Mustard	0	1	0	0
Turnip	0	0	19	1
Chinese cabbage	0	0	16	0

Table 3

Summary of reactions produced in six plant species, inoculated with strains of four groups of pv. *maculicola* and pv. *tomato*

	tomato	cauliflower	radish	turnip	cucumber	coronatine-like activity
group I	+a)	+	V	V	V	V
group II	+	+	+	-	-	-
group III	+	+	+	+	V	+
group IV	+	+	+	-	+	+

a) symbols used :

+ : formation of leaf spot (on tomato, cauliflower, radish, turnip or cucumber) or positive coronatine-like activity on potato tuber slice

- : no reaction

V : variable results depending on strains tested.

All the 173 strains tested were pathogenic both on tomato and on cauliflower, causing numerous necrotic spots on leaves. The reactions on radish, turnip, cucumber and potato slices varied among the groups or the strains (table 3). Several strains which proved to have coronatine-like activity tended to produce lesions with chlorotic halo on susceptible plants. The other strains showed symptoms indistinguishable from each other on each plant species inoculated, with the exception of the group IV strains which produced large coalescent necrotic lesions on radish plant. The symptom on cucumber plants were indistinguishable from that caused by pv. *lachrymans*; exudating bacterial ooze from the lesions under humid conditions.

DISCUSSION

Based on phenotypic characteristics, it has been suggested that the strains of pv. *tomato* and of pv. *maculicola* resemble closely to each other (HILDEBRAND *et al.*, 1988). We further demonstrated that the phenotypic properties divided pv. *maculicola* and pv. *tomato* into four groups. However, the division did not correspond to the pathovar designation of each strains. For example, the strains isolated from cauliflower, designated to pv. *maculicola*, distributed into three groups. Furthermore, the pathotype strain of each pathovar belonged to the same group i.e. group I.

Our inoculation experiments showed that the strains of both pathovars were pathogenic on both tomato and cauliflower. Although some of the strains were proven to be pathogenic on cucumber, pv. *lachrymans* was pathogenic on neither tomato nor cruciferous plants and easily distinguished from pv. *maculicola* or pv. *tomato* by several phenotypic characteristics (data non shown). The pathotype strain of pv. *maculicola* (ICMP 3935) failed to infect cucumber and to produce coronatine-like activity, while the pathotype strain of pv. *tomato* (ICMP 2844) infected cucumber and produced coronatine-like activity. However, one of the other strains from cauliflower (90 S4 ; Japanese field isolate) infected cucumber and produced coronatine-like activities, while some stains of pv. *tomato* (eg. ICMP 2843, 3361) did not. Recently, it has been suggested that several strains of the two pathovars were pathogenic in various degrees on tomato and on several cruciferous plants including *Arabidopsis thaliana* (WHALEN *et al.*, 1991). These results indicate that the two pathovars can not be discriminated by either phenotypic or pathological terms.

DENNY (1988) reported that the strains of pv. *tomato* and pv. *maculicola* were indistinguishable by RFLP analysis. Her report also support our conclusion.

Furthermore, our preliminary DNA-DNA hybridization analysis showed 93 to 97% homology between the pathotype strains of the two pathovars.

Consequently it is impossible to assign an isolate of this group to either one of the two pathovars, if it is not known from which plant it was isolated. *P. s.* pv. *maculicola* and *P. s.* pv. *tomato* are synonymous. The name *Pseudomonas syringae* pv. *maculicola* has priority and we propose that it be retained, with ICMP 3935 as the pathotype strain.

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Plasmid profiles and transposon mutants in *Pseudomonas syringae*

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ABSTRACT

The presence of plasmids in *P. syringae* strains isolated from different geographical regions was studied. Plasmid DNA was detected in pathovars : *aptata*, *atrofaciens* where it was not observed before. Molecular sizes of plasmids ranged from 4.5 to 135 kb. The occurence of the plasmids in *P. syringae* cells did not correlate with the distribution of the bacteria to serogroups by O-antigen principle. Mobilization transfer of plasmid from *P.s.* pv. *atrofaciens* strains by RP 4 (Inc P-1) has failed. It was established that both virulent and avirulent *P. syringae* strains contained plasmids. *P.s.*pv.*phaseolicola* mutants have been obtained by transposon mutagenesis. Transposition frequency was higher in recipient strains carrying plasmid with a molecular size of 135 kb. The frequency of auxotrophic mutants makes up 0.3 %.

KEYWORDS

Bacteria, serogroup, plasmid, conjugation, transconjugant, mutant, antibiotic, *Pseudomonas syringae.*

Pseudomonas syringae causes considerable losses in agriculture, damaging major groups of agricultural plants.

The purpose of our research was to study the plasmids spreading in *P. syringae* strains having different antigenic affinity and recipient characteristics; to select the proper system for transposon mutagenesis and to obtain transposon mutants of *P.s.* pv. *phaseolicola.*

MATERIALS AND METHODS

36 strains of *P.s.*pv.*atrofaciens* were provided by Dr. Pasichnic and 47 strains of *P. syringae* were obtained from the Collection of phytopathogenic bacteria of Institute of Microbiology and Virology of Academy of Scienses, Ukraine (IMV ASU). These strains are listed in the Table. They belong to 9 serogroups (PASTUSHENKO & SIMONOVICH,1979). Strains retained the names given at the original isolation.

MPA medium (BELTUCOVA et al.,1968) and LB medium (MANIATIS et al.,1982) were used to culture bacteria. M9 minimal medium was used for the screening of auxotrophs (ZUKOV-VERESHNIKOV PEKHOV, 1963).

Resistance to antibiotics was investigated by serial dilution plating techniques. Plasmid DNA isolation was carried out by procedures of BIRNBOIM and DOLY (1979) and KIESER et al. (1984).

The molecular size of plasmids was determined by comparing their mobility at electrophoresis in agarose gel to the mobility of the plasmids of known molecular sizes : R68, RP8, R7K, pSUP5011, RSF1010, pUC19.

Bacterial conjugations between *E. coli* and *P.s.phaseolicola* recipient strains were performed by the method of ANDERSON and MILLS (1985). Donor plasmids pSUP2021, pSUP5011 (SIMON et al.,1985), COL1b::Tn5 offered by Dr. Khmel (IMG, Russian Academy of Scienses, Moscow) and pJBJ4I::Tn5 - from Dr. Didyk (IMV ASU) have been used for transposon mutagenesis.

RESULTS AND DISCUSSION

Plasmids were found in 42 out of 82 collection strains of *P. syringae*. The absence of plasmid DNA in strains of the pathovars: *cerasi f. pyri, morsprunorum, maculaicola, vignae, wieringae* (1, 11 and 1V serogroups) may be due to the low number of the strains being studied.

Plasmids from strains of *P. syringae* pvs *aptata* and *atrofaciens* (serogroups 1,11,1V,V1) have been isolated for the first time.

Molecular sizes of plasmids from *P. syringae* cells, ranged from 4.5 to 144 kb (Table 1). In some strains presenting serogoups 1,1V,V1,1X, were present 3-4 plasmids. Virulent strains of different serogroups were either plasmid-containing and plasmid-free.

The resistance of *P.s.*pv.*atrofaciens* strains to rifampicin, trimethoprim, chloramphenicol, nalidix acid was 100 mg/ml and higher. Strains resistant to ampicillin, erytromycine, tetracycline, streptomycin and kanamycin have not been discovered.

CHARACTERISTICS OF COLLECTION STRAINS OF P.SYRINGAE

Pathovar	Number of strains	Sero-group	Virulence	Strain origin	Molecular size of plasmid (kb)
Aptata	1/1	1V	+	Beetroot, Ukraine	75
Atrofaciens	36/22	1,11 1V, V	+	Wheat, rye, Ukraine	4.5-105
Glycinea	1/1		+	Soy-bean, Ukraine	90-135
Lachrymans	4/2	1X	+	Cucumbers, Ukraine	6.0-144
Morsprunorum	1/0		*	Fruit-trees, Ukraine	
Phaseolicola	12/6	V1	+	Haricot, Ukraine	30-135
Pisi	3/3	1V	+	Pea, Ukraine	36-67,5
Syringae	6/2	1,11 111	+,-	Fruit-trees, Ukraine,Italy, England	120-135
Cerasi	2/1	11		Fruit-trees, Ukraine	6-7,5
Cerasi f. Pyri	1/1	11	*	Poplar,Ukraine	
Holci	7/1	11	+,-	Sorgnum,sudan-grass,Ukraine, Yugoslavia	3-135

Note: ^ - ratio of total number of strains and plasmid containing strains;
x - the names o pathovars are given as presented in Simonovich, Pastushenko (1979);
+ - virulent;
- - avirulent;
* - not checked.

Recipient characteristics of *P.s.*pv.*atrofaciens* have been studied in the experiments on conjugation with donor strains of *E. coli* C600 (RP4, IncP-1). Frequency of plasmid transfer into *P.s.*pv.*atrofaciens* ranged from 10^{-7} to 10^{-6} per donor cell, and back transfer from *P.s.*pv.*atrofaciens* strains into *E.coli* 10^{-3} to 10^{-2}.Frequency of plasmid RP4 transfer into strains of *P.s.*pv.*atrofaciens* was from 10^{-7} to 10^{-6}. However,mobilization transfer of resident plasmids into these strains has not been noticed.

Plasmid-free strain 4012 and plasmid-containing strain 4270 of *P.s.*pv.*phaseolicola* served as recipients for transposon mutants. It is notable that Km transconjugants of plasmid-free *P.s.*pv.*phaseolicola* 4012 have been obtained with a frequency of 10^{-7} per donor cell after mating with *E.coli* S17-1 (pSUP5011) while the frequency of obtaining mutants of plasmid-containing *P.s.*pv.*phaseolicola* strain 4270 was higher and came up to 10^{-5} per donor cell. About 3000 transposon mutants of *P.s.*pv.*phaseolicola* have been obtained, the frequency of auxotrophic mutants among them accounted for 0.3% of the total number.

Among donor Tn5-containing plasmids for transposon mutagenesis the system constructed by SIMON *et al.*,1983) - pSUP2021, pSUP5011 was the most effective. It provided the frequency of appearance of Km transconjugants of *P.s.*pv.*phaseolicola* from 10^{-5} to 10^{-6}, since the frequency of such clones appearance with the use of suicidic plasmids COL1b:: Tn5 and pJB4JI:: Tn5 was not higher than 10^{-7} to 10^{-8}. It is notable that the appearance of auxotrophic mutants of one type did not prevail; a wide diversity of mutants according to their varioux metabolic needs have been obtained.

By using donor pSUP-plasmids nearly 1000 Km transconjugants of *P.s.*pv.*atrofaciens* have been obtained and checked for pathogenicity. However, avirulent strains were absent among them.

The experiments on the construction of the original system for transposon mutagenesis of *P.syringae* on the basis of pSUP-plasmids and lux-genes from photobacteria are being carried out. They are supposed to facilitate the mutant receiving procedure.

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Application of the polymerase chain reaction (PCR) for enhanced detection of *Xanthomonas campestris* pv. *citri*

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ABSTRACT Plasmid pFL62.42 contains a 4.2 Kb *Bam* HI fragment cloned from plasmid DNA of strain XC62 of *Xanthomonas campestris* pv. *citri* (*Xcc*) and is a highly sensitive and specific hybridization probe for *Xcc*. Plasmid pFL1 contains a 562 base pair internal *Eco* RI fragment from pFL62.42 and gives slightly more specific results. Both plasmids detected 44/44 pathotype A strains of *Xcc* originally isolated in 15 countries. Nearly all strains of pathotypes B and C were also detected with these probes. Neither probe reacted with strains of *X. campestris* isolated from symptomless *Citrus* nor with 56 strains of *X. campestris* associated with citrus bacterial spot disease (CBS) in Florida citrus nurseries nor with bacteria from other genera. The sensitivity in dot blot assays using chemiluminescent detection was approximately 2-7 ng DNA/spot. Probe pFL1 specifically detected Asiatic strains of *Xcc* in extracts of leaf lesions. Nucleotide sequence information from pFL1 was used to design primers in order to apply the polymerase chain reaction (PCR) for the detection of *X. c. citri*. Seven 18 bp oligonucleotide primers were designed and tested with DNA from *X. c.* pv *citri* and other strains of X. campestris associated with *Citrus* as templates in PCR reactions. Primer pair 2 / 3 directed specific amplification of target DNA of pathotype A of X. c. pv *citri*, but not of other pathotypes of *X. c.* pv *citri*. A buffer of pH 9.0 which contained 1 % Triton X-100 and 0.1 % gelatin was absolutely required for successful amplification of the GC rich target DNA.

KEYWORDS : Diagnostic Probe, Citrus Bacterial Canker, Pathogen Detection

Recent outbreaks of Citrus Bacterial Canker (CBC) in Florida, USA have stimulated a great deal of research into the biology of the pathogen *Xanthomonas campestris* pv *citri*. (reviewed by Stall and Civerolo, 1991). Because the pathogen is the target of international quarantine efforts (Anonymous, 1991) development of

rapid and reliable diagnostic procedures for this pathogen has been a priority. This task has been complicated by the presence of another distinct disease in Florida Citrus nurseries, citrus bacterial spot (CBS), caused by other strains of *Xanthomonas campestris* (Schoulteis it al., 1987). Although the taxonomic position of *Xanthomonas campestris* pv *citri* has been controversial (Gabriel et al., 1989; Young et al. 1991), there is widespread agreement that the strains of *X. campestris* which cause CBS are quite distinct from, and should not be confused with any of the several pathotypes (A-D) causing CBC.

We and others have shown that RFLP analysis of genomic DNA of these strains can separate the strains into groups which are entirely consistent with groups independently derived from other data (Gabriel et al. 1988; Hartung and Civerolo, 1989). This work was recently extended by a RFLP analysis of plasmid DNA from strains of *X.c.* pv *citri*. (Pruvost et al., 1992). During the course of this study, a 4.2 kb *Bam* HI fragment was found in 85.2% of the most pathogenic strains (Asiatic or CBC-A) of *X.c.* pv. *citri*. After molecular cloning from strain XC62 as pFL62.42, this fragment distinguished subtypes of *X. c.* pv. *citri* in Southern blots and importantly, did not cross react with strains of *X. campestris* which cause CBS.An internal 562 bp *Eco*RI fragment was cloned as pFL1 and the two fragments used to develop a rapid and sensitive dot blot assay for *X. c.* pv *citri* (Hartung, 1992).

The Polymerase Chain Reaction (PCR) (Saiki et al. 1988) allows rapid, specific and sensitive detection of DNA sequences, and thus is ideally suited for the detection of plant pathogens. We report the development of a PCR based assay for X. c. pv citri based on the DNA sequence of the *Eco*RI insert in pFL1.

MATERIAL AND METHODS

The *Eco*RI insert from pFL1 (Hartung 1992) was cloned into the sequencing vectors M13mp18 and M13mp19 by standard methods. The complete nucleotide sequence of both strands of the insert was determined by dideoxy sequencing using the Sequenase system (United States Biochemical, Cleveland, Ohio). Oligonucleotides 18 bp in length were designed using the computer program Nuc-it (Compu-Right, Gaithersburg, MD). Paired primers were selected which had low homology to other sequences in the target fragment and which had closely matched calculated thermal melting points. PCR assays were performed in a DNA Thermal Cycler (Perkin Elmer/Cetus) in 50 ul reactions which typically contained 50 ng genomic DNA, dNTP's at 200 uMolar and primers at 1 uMolar each. Three

reaction buffers were used: 10 mM Tris/Cl (pH8.3), 50 mM KCl, 3 mM Mg^{++} (Buffer I), Buffer I with 3% formamide and 7% glycerol (Buffer II) and 50 mM Tris/HCl (pH9.0), 20 mM NaCl, 1% Triton X-100 and 0.1 % gelatin and 3 mM Mg^{++} (Buffer III) (Barry et al., 1991). Denaturation was at 95 C for 70 seconds, annealing was at 45-65 C for 60 seconds and extension was at 72 C for 1 minute plus 2 seconds per cycle for 30 cycles. Aliquots containing 25% of the reaction were removed and subjected to agarose gel electrophoresis in 3% composite agarose gels (3 parts Nusieve : 1 part standard LE agarose (FMC, Rockland ME)) or 1.5 % LE agarose gels. The oligonucleotide primers were synthesized commercially (Genosys, The Woodlands, Texas). The bacterial strains used were described previously (Hartung, 1992).

RESULTS AND DISCUSSION

Because the DNA sequence of the 562 bp target fragment was 60.6 % G+C, and the target sequence was part of a plasmid, difficulty in achieving amplification was anticipated. Because buffer composition can effect PCR results, the standard PCR buffer recommended by Perkin Elmer/Cetus was compared to the same buffer supplemented with the cosolvents glycerol and formamide (Sarkar et al. 1990; Smith et al., 1990) and to a third buffer (Barry et al. 1991). Primer pairs 2/3, 4/5, 6/7 and 1/5 were expected to prime amplification of products of 222 bp, 462 bp, 478 bp and 261 bp respectively when homologous (XC62) DNA was used as the target template (Figure 1). No amplification of homologous target DNA was achieved using Buffers I or II at any annealing temperature from 45 C - 65 C except for primer pair 1/5 which successfully primed amplification of its target sequence when annealed at 65 C. In contrast, specific amplification products were produced in Buffer III at all annealing temperatures from 45 C to 65 C with all four primer pairs. The results from the 55 C annealing reaction are typical (Figure 2). Specificity was improved when the annealing temperature was 60 C.

Primer pair 2/3 was used to amplify target sequences in genomic DNA from 12 CBC-A strains originally isolated in 12 countries. This primer pair did not find target sequences in DNA from 5 strains of pathotype B,C and D of *X.c.* pv. *citri* (Figure 3) nor in DNA from 4 strains of *X. campestris* associated with CBS. A product was found when DNA from a single strains of *X. c.* pv *vignicola* and *X.c.* pv *bilvae* were tested. These results are consistent with hybridization analysis of these same strains (Hartung, 1992), in which pathotype B, C and D strains gave

consistently weaker results than pathotype A strains in dot blot assays, and produced homologous bands of different size in Southern blots.

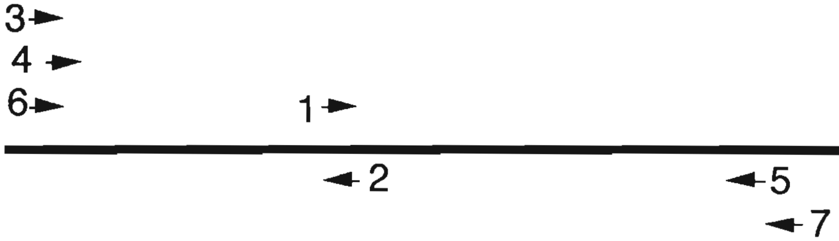

Figure 1. Diagram of the 562 bp target region for PCR amplification of *X. c.* pv. *citri* DNA showing the relative positions and orientations of the 18 bp oligonucleotide primers 1-7.

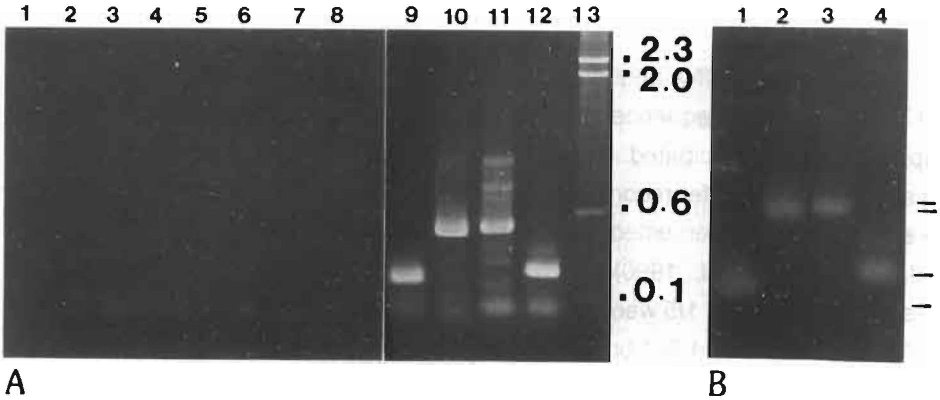

Figure 2 (A) Successful amplification of target DNA is dependent on the reaction buffer. Lanes 1-4: Buffer I; Lanes 5-8; Buffer II; Lanes 9-12: Buffer III. Primer pair 2/3 was used for samples 1,5 and 9; pair 4/5 for samples 2,6 and 10; pair 6/7 for samples 3, 7 and11 and pair 1/5 for samples 4,8 and 12. Annealing temperature was 55 C. **(B)** Same as Fig. 2 (A), lanes 9-12 except the annealing temperature was 60 C. The size of Lambda/*Hin* dIII standards is given in the margin. The template DNA was 50 ng of *Eco*RI digested pathotype A (XC62) DNA for all reactions.

Although amplification was successful with genomic DNA of pathotype A of *X. c.* pv *citri* , yield was improved if the DNA was digested with *Bam* HI or *Eco* RI prior to amplification *(not shown)*. This is probably due to the supercoiled (plasmid) state of the target DNA. However amplification of target DNA was successful when starting with intact bacteria *(not shown)* as described previously for other species (Barry et al., 1991). The composition of the reaction buffer was critical for successful amplification of *X. c.* pv *citri* DNA. Buffer III differs from the standard

PCR buffer by having a higher pH and by incorporating 1% Triton X-100 and 0.1% gelatin. We do not know which of these ingredients is most responsible for the success of the amplification.

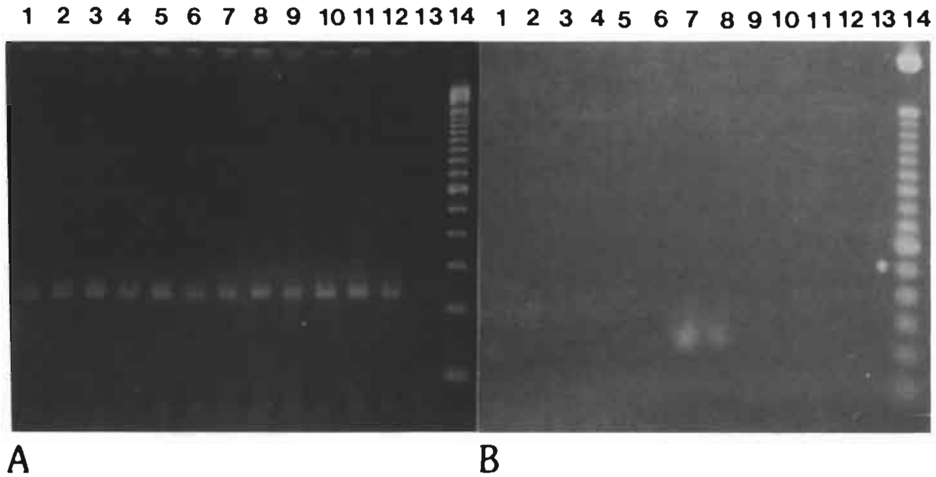

Figure 3 Specific amplification of target in DNA from pathotype A of *X.c.* pv. *citri* by the the polymerase chain reaction. **(A)** Template DNA was from pathotype A strains of *X. c.* pv. *citri* from 12 countries. **(B)** Template DNA was from pathotype B,C and D strains of *X.c.* pv. *citri* (lanes 1-5), pvs *alfalfae, bilvae* and *vignicola* (lanes 6-8), and Florida CBS strains (lanes 9-12). The no DNA control reaction was in lanes 13. Primer pair 2/3 was used, and annealing was at 58 C. The BRL 100 bp ladder was run in lanes 14, with the lowest band of 100 bp. The position of the predicted 222 bp product is marked in the margin.

Pathotypes B,C and D are considered less virulent than pathotype A of *X.c.* pv *citri*, are much less widely distributed (Stall and Civerolo, 1991), and so pose less of a threat to the citrus industry. Thus the clear detection of pathotype A and non-detection of Pathotypes B,C and D represents a useful complement to the previous dot blot assays (Hartung , 1992). None of the primer pairs found targets in CBS strains tested. Experiments are in progress to determine the level of sensitivity which can be achieved with this assay system. Pathotype A has also been detected directly in washes from citrus canker lesions with this PCR technique.

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Efficient detection of *Erwinia amylovora* by PCR-analysis

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ABSTRACT

Detection and identification of the fireblight pathogen, *Erwinia amylovora*, can be accurately done by PCR-analysis in less than 6 hours. Two oligomers derived from a 29 kb plasmid, which is common to all strains of *E. amylovora*, were used to amplify a 0.9 kb *Pst*I-fragment of the plasmid. By separation of the PCR-products on agarose gel, this fragment was only detected when *E. amylovora* DNA was present in the amplification assay. It was not found when DNA from other plant-pathogenic bacteria was used for the assay. A band specific to the 0.9 kb fragment was still produced with DNA from less than 100 *E. amylovora* cells. A signal of similar strength was also obtained from *E. amylovora* cell lysates in the presence of the mild detergent Tween 20. Signals were weaker when bacteria were added to the PCR reaction mixture without the detergent. As with results obtained from hybridization experiments using pEA29 DNA, the PCR signal was obtained with *E. amylovora* isolates from various geographic regions. This technique is also useful for detection of the fireblight pathogen in extracts of infected plant tissue, e.g. bark, leaves and pollen grains.

Keywords: polymerase chain reaction, fireblight, field samples

INTRODUCTION

The Gram-negative bacterium *Erwinia amylovora* causes the disease fireblight on pome fruit trees and other rosaceous plants (VAN DER ZWEET & KEIL, 1979).

Historically, fireblight was restricted to the north-east part of North America with its occurence first described more than 200 years ago. In this century epidemic spread has been reported from widely separated places all over the world. Although the long distance spread has not been unambiguously correlated to specific events, it seems that plant material appears to be the most important source for fireblight epidemics, which may be confined by monitoring suspect plants lacking disease symptoms. Conventional diagnostic methods, including semiselective media (MILLER & SCHROTH, 1972) or immunfluorescence techniques (ROBERTS, 1980), cannot detect small quantities of *E. amylovora*. Therefore, we have developed a fast, sensitive and species specific PCR-detection system that is based on information from colony hybridization experiments using the *E. amylovora* plasmid (pEA29) and its cloned fragments as probes (FALKENSTEIN et al., 1988). Results from these experiments indicated that there is no known homology of plasmid pEA29 to DNA of other plant-pathogenic bacteria. The plasmid is involved in the bacterial thiamin metabolism (LAURENT et al., 1989) and its curing from *E. amylovora* causes a delay in development of disease symptoms (FALKENSTEIN et al., 1989). This may be the reason why plasmid free bacteria are not present in natural populations of the pathogen. To avoid the time-consuming and labor-intensive procedure of colony hybridization, we have used DNA amplification by PCR (ERLICH, 1989) for detection of *E. amylovora.* For this purpose we cloned the 0.9 kb PstI-fragment of pEA29 into a pfd-plasmid (GEIDER et al., 1985) and confirmed by colony hybridization that the insert was specific for *E. amylovora*. The fragment was sequenced at both ends and two oligonucleotide sequences were synthesized in order to amplify DNA by PCR.

MATERIAL and METHODS

Bacterial strains and plasmids. All strains used for PCR-analysis were from our laboratory collection. *Erwinia amylovora* strains Ea1/79, Ea5/84, Ea11/88, EaX11/88, CFBP1430, Ea273, Ea1496/66, T91; *Erwinia herbicola* strains 2035 and NZ; *Erwinia carotovora* subsp. *atroseptica* strain 185; *Agrobacterium tumefaciens* strain C58; *Pseudomonas syringae* pv. *syringae* strain 2.

The 0.9 kb PstI-fragment of pEA29 was cloned into the plasmid pfdB14Z', which contains the origin of replication from bacteriophage fd, the Cm-resistance gene and the lacZ'-gene (GEIDER et al., 1985).

Primers and conditions for PCR. Two 17mer oligonucleotide sequences from the borders of the pEA29 PstI-fragment were chosen as primers for amplification by PCR. Primer A: CGGTTTTTAACGCTGGG; Primer B: GGGCAAATACTCGGATT.

PCR reactions were carried out in a volume of 50 μl containing 25 pmoles of each primer and 0.5 U of *Tth*-polymerase. The buffer system and the cycling conditions are described elsewhere (BERESWILL et al., 1992). A $MgCl_2$ concentration of

1.5 mM and an annealing temperature at 52 °C produced best results. PCR products were analysed by agarose gel electrophoresis.
Sample preparation for PCR. Phenol extracted DNA was prepared after lysozyme/ SDS treatment of cells according to standard procedures (SAMBROOK et al., 1989). Bacterial Cells were grown overnight at 28 °C in 10 ml Standard I nutrient broth (Merck), centrifuged, washed and resuspended in an 0.6 % NaCl-solution. Leaves and bark samples were placed in an Eppendorff-tube and partially homogenized with an Eppendorff-pipette-tip in 100 µl 0.6 % NaCl solution. A 1 µl aliquot of the homogenate was then added without further treatment to the PCR reaction mix. Pollen grains were collected in a pollen-trap and resuspended (20 g/100 ml) in 0.6 % NaCl solution. This suspension was shaken overnight at 4 °C and diluted. An aliquot (10 µl) of the dilution series added directly to the PCR reaction mixture. Wash-solutions from other plant tissues were analysed after shaking sample material overnight in sterile 0.6 % NaCl solution at 4 °C and pelleting resuspended bacteria by centrifugation at 15.000 rpm in a Sorvall HB4-rotor.

RESULTS

Sensitivity of the PCR system.
Under optimal conditions PCR amplification of 10 ng *E. amylovora* DNA gave rise to an intensive band representing the amplified 0.9 kb *Pst*I-fragment of plasmid pEA29. In a Southern blot this band hybridized to the corresponding fragment from the *E. amylovora* plasmid. When the DNA concentration was lowered, the amplification product was still seen for 1 pg DNA corresponding to 100 bacteria.

Species-Specifity of the PCR-system.
PCR-analysis of DNA from other plant associated bacteria like *E. herbicola* strains NZ and 2035, *E. carotovora* subsp. *atroseptica* strain 185, *Pseudomonas syringae* pv. *syringae* strain 2 revealed unspecific PCR products that were often shorter than 0.9 kb. Probing DNA of *Escherichia coli*, *E. carotovora* subsp. *carotovora*, *E. chrysanthemi* and from *Agrobacterium tumefaciens* also failed to give a PCR-band at 0.9 kb. Lowering the primer concentration to 2 pmoles each or raising the annealing temperature over 52 °C abolished unspecific amplification products but reduced sensitivity of the assay.

PCR-detection of whole cells and E. amylovora isolates from various gegraphic regions.
The PCR-system described here is also suitable for the detection of intact cells from liquid cultures or from agar plates. The presence of 1 % of Tween 20 in the reaction mixture (KANG et al., 1991) increased sensitivity to 30-50 colony forming units. Specifity was demonstrated for detection of 500 *E. amylovora* cells in the

presence of a 100 fold excess of cells from other plant associated bacteria like *E. herbicola* NZ, *E. carotovora* subsp. *atroseptica* 185, *A. tumefaciens* C58, *P. syringae* pv. *syringae* 2. PCR-analysis of *E. amylovora* strains from different geographic regions revealed that cells from all isolates produce an amplification signal at 0.9 kb. This indicated the presence of the 0.9 kb *Pst*I-fragment of pEA29 in various *E. amylovora* strains.

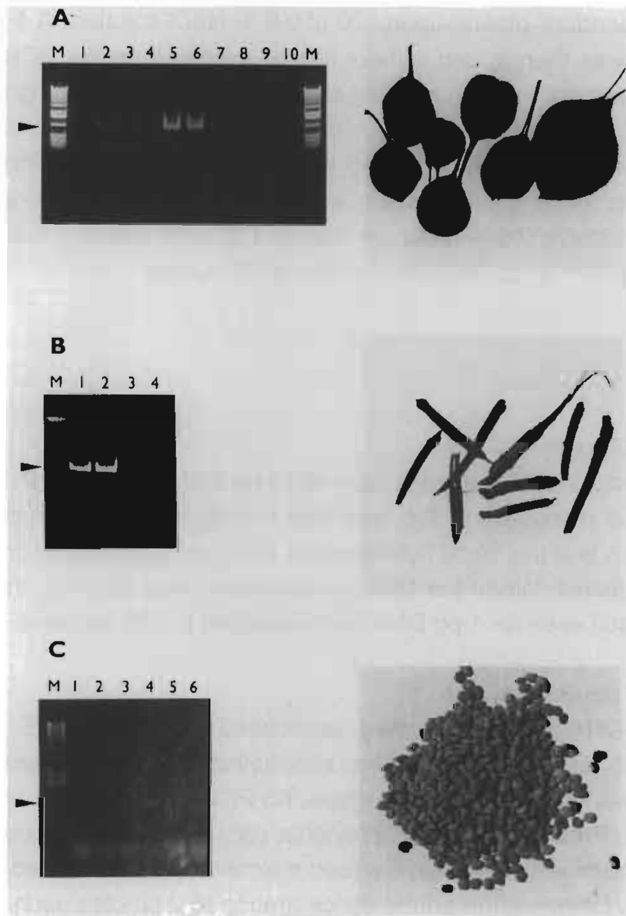

Fig. 1: PCR-analysis of plant material for presence of *E. amylovora*. Panel A: Samples from leaves with fireblight symptoms. 100 mg tissue, homogenized in 100 µl 0.9 % NaCl; 1 µl applied. Lanes 1,4,7: undiluted; 2,5,8: 10^{-1} dilution; 3,6,9: 10^{-2} dilution. Lanes 1-3: sample from center of leaf necrosis; 4-6: from transition zone green/necrotic; 7-9: from green leaf tissue; 10: negative control. Panel B: Samples from bark of an apple seedling. 1 g bark was extracted with 10 ml 0.9 % NaCl. The suspension was pelleted and resuspended in 100 µl 0.9 % NaCl. 10 µl were applied to PCR; lanes 2,4: diluted 10^{-1}. Lanes 1,2: material from old canker; 3,4: from a healthy leaf of the same seedling. Panel C: Samples from pollen suspensions (2 g/10 ml 0.9 % NaCl); 1 µl applied. Lanes 2,4,6: diluted 10^{-1}; 1,2: from pome fruits orchard without fireblight symptoms; 3,4: from experimental orchad with fireblight; 5,6: from orchad without disease, samples were drawn a week later then for assay in lanes 1 and 2. ►, position of 0.9 kb signal.

Specific detection of E. amylovora under field conditions.

The PCR-detection system here can easily detect *E. amylovora* in wash-solutions from plant material (see Fig. 1) or obtained from an air sampler (EHRIG & FICKE, 1990). But it is also possible to identify the fireblight pathogen directly in plant tissue samples without extraction. We therefore used leaves and pollen grains from plants previously infected with *E. amylovora* and analysed the samples directly in PCR. An intensive band at 0.9 kb identified the presence of the pathogen and the usefulness of the detection system under field conditions (see Fig. 1). Because unknown contaminants present in plant material inhibit the polymerase, it is recommended to analyze samples in dilution series.

DISCUSSION

Long distance as well as local spread of fireblight, caused by transfer of plant material contaminated with *E. amylovora*, could be considerably minimized by monitoring such material for the pathogen using sensitive detection techniques. Serological techniques (ROBERTS, 1980) including monoclonal antibodies (LIN et al., 1987) or semiselective media (MILLER & SCHROTH, 1972) can be applied in the case of massive contaminations. Latent infections with low concentrations of the pathogen require a more sensitive detection method. Total genomic DNA of *E. amylovora* has been applied for detection of the pathogen (HALE & CLARK, 1990). Considering homology among the genomes of Gram negative bacteria, the signal obtained might be ambiguous and especially at low intensity, not indicative for occurence of fireblight. DNA from the 29 kb plasmid, common to *E. amylovora* strains, appears to be very specific as probe in hybrdization experiments (FALKENSTEIN et al., 1988). The use of oligonucleotide primers derived from the 0.9 kb PstI-fragment of plasmid pEA29 in PCR provided a sensitive and specific tool for detection of the fireblight pathogen. We found an easily detectable signal for the presence of 50 bacteria representing approx. 150 plasmid copies. We consider this a practical detection limit for the PCR assay described here. PCR applications for detection of bacteria in natural samples (LAMPEL et al., 1990; STEFFAN & ATLAS, 1991) and of pathogenic viruses have been described (INNIS et al., 1990). We circumvented labor intensive hybridization techniques by direct detection of the PCR product specific for *E. amylovora* by gel electrophoresis. Since intact bacteria could be detected in plant material even in the presence of other bacteria, our PCR system might be useful for many practical purposes. The fireblight pathogen E. amylovora may thus be monitored in latent infections of pome fruit orchards, in nurseries, in exported fruits and in other host plant material.

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Identification and characterization of new pathogenic *Agrobacterium tumefaciens* strains

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Three new strains of *Agrobacterium tumefaciens* were isolated, which incited formation of tumors producing unknown opine-like molecules. These opine-like molecules resemble the opines agropine, mannopine and agropinic acid, but could be distinguished from them by their biological properties. The first molecule, termed chrysopine, is specifically degraded by the inciting pathogenic agrobacteria. The second one, called chrysopine II, resembles agropinic acid, and is degraded by strains utilizing agropinic acid. Interestingly, the third one, santhopine, is degraded by most of the pathogenic and non-pathogenic agrobacteria.

Key words: *Agrobacterium tumefaciens*, Ti plasmid, opine.

INTRODUCTION

Agrobacterium tumefaciens is the causative agent of crown gall, a proliferative disease affecting most of the dicotyledonous plants. In the bacteria, the pathogenic element is the Ti plasmid. By transferring a part of the Ti plasmid DNA to the nucleic genome of the host plant, the bacteria modify the mitotic and metabolic activities of the plant cells. Plant cells multiply to form a tumor, and their metabolism is redirected towards production of unusual low molecular weight molecules called opines. The opines are released in the intercellular spaces of the tumor, where they are used as growth substrates by the inciting agrobacteria. Additionally, some of the opines induce the conjugal transfer of the pathogenic Ti plasmid from a virulent strain to an avirulent recipient. These features led to the formulation of the opine concept and the genetic colonization

theories which both describe the crown gall tumor as ecological niche where the presence of opines favours growth and dissemination of bacterial pathogenicity (reviews: DESSAUX *et al.*, 1992; GELVIN, 1992; WINANS, 1992).

Currently, eight types of Ti plasmids and over twenty opines are known. The classification of pathogenic plasmids is based on the nature of opines synthesized in tumors, and degraded by the bacteria. This classification is in constant evolution since new opines and new types of plasmids are isolated. In this paper we describe the discovery of three new opine molecules and at least one new type of Ti plasmid.

MATERIALS AND METHODS

Bacterial strains. Strain ANT 4 was isolated from a *Chrysanthemum* tumor. Strains 3.10 and 3.44 were isolated from a *Ficus* tumor. *Agrobacterium* strains C58 is a wild-type nopaline-type tumor harboring two plasmids: the pathogenic plasmid pTiC58 and the cryptic megaplasmid pAtC58. Strain C58C1 is a pTi-free derivative of C58. Strain C58C1pAt$^-$ is a pAt-free derivative of C58C1. All other *Agrobacterium* strains are wild-type isolates. Some are non pathogenic (see text). The virulent strains harbor various Ti plasmids (see text or figure legends).

Bacterial growth media. Bacteria were routinely cultivated on modified LB (5 g/l NaCl) as a rich non selective medium. AT medium was used as a minimal medium (PETIT & TEMPE, 1978). Liquid cultures were shaken on a rotary shaker. Plates and liquid cultures were grown at 26 to 28°C.

Chemicals. All chemical were from commercial sources except mannityl opines which were synthesized in the laboratory using standard procedures (PETIT *et al.*, 1983).

Pathogenicity assays. Bacteria were grown for 24 to 48 hours on solid LB medium. Pathogenicity assays were performed on *Datura stramonium*, *Kalanchoe tubiflora*, *K. daigremontiana*, and *Nicotiana tabaccum* plants. Plants were wounded with a sterile scalpel blade and inoculated on stems and apex. Results were observed by visual inspection 4 to 6 weeks after inoculation. When necessary, samples of the tumors were taken from the plant and further analyzed for presence of opines.

Opine detection and utilization assays. Opines were detected in tumors using high voltage paper electrophoresis (HVPE) at pH 1.9 (PETIT *et al.*, 1983) and at pH 2.8. The latter buffer was made by adding sufficient amount of concentrated NaOH to the pH 1.9 buffer. Opine utilization by bacteria was assessed by analyzing disappearance of these molecules from bacterial culture supernatants. Culture media (200 µl) was AT minimal medium supplemented with ammonium sulphate (1 g/l), yeast extract (100 mg/l), and with the following opines: nopaline (2.5 mM), octopine (2.5 mM), ridéopine (2 mM), leucinopine (2.5 mM), cucumopine (2 mM), mikimopine (2 mM), mannopine (2 mM), agropine (2 mM) and agropinic acid (2 mM).

Biological purification of tumor extracts. Tumors (*ca.* 30 g, fresh weight) were sliced in thin pieces, placed in a beaker, submerged with distilled water, and moderately heated (100°C, 1 min.). Treated tissues and solution were filtered through glass wool, and the resulting filtrate was centrifuged (30,000 x g) for 30 min. at 4 °C. After filtration of the supernatant through 0.45 µm ultrafiltration membranes, the resulting medium was inoculated with strain C58C1pAt$^-$. Bacteria were grown for 2 to 4 days and the disappearance of amino acids and sugars was monitored by high voltage paper electrophoresis. The purification step was terminated by centrifugation of the medium (10,000 x g, 30 min., 4°C) and filtration through 0.45 µm ultrafiltration membranes.

Plasmid analysis. Plasmid contents of *Agrobacterium* strains was analyzed using the Eckhardt procedures in 0.8% agarose vertical gels (ECKHARDT, 1978).

RESULTS AND DISCUSSION

Identification of new pathogenic strains. Strains ANT 4, 3.10 and 3.44 were originally isolated from tumors formed on *Chrysanthemum* and *Ficus*. Following purification of these strains, pathogenicity was investigated by inoculating bacteria on various test plants (see Materials and Methods). These three strains induced tumors on all tested plants and should therefore be regarded as pathogenic. Their ability to degrade opines was analyzed by inoculating these strains and other control strains to a degradation assay medium which contained

opines representative of all known types of Ti plasmids. Samples were removed at different times following inoculation and analyzed by high voltage paper electrophoresis. Results (fig. 1) indicate that the new isolates 3.10 and 3.44 were able to degrade only nopaline. Remarkably, strain ANT 4 failed to catabolize any of the known opines except leucinopine (data non shown).

Identification of new opine-like molecules in tumors induced by strains ANT 4, 3.10 and 3.44. To check the opine-type of strains 3.10 and 3.44 and the unusual catabolic properties of strain ANT 4, the presence of opines was investigated in tumors induced by the three new isolates. Indeed, nopaline was detected in tumors induced by either strains 3.10 and 3.44. However, using silver nitrate reagent, three additional compounds were detected in ANT 4, 3.10 and 3.44 tumor extracts that were not present in uninfected tissues (not shown). Different observations strongly suggest that the structures of these three opine-like molecules (called chrysopine, santhopine and chrysopine II) should be related to those of, respectively, agropine, mannopine and agropinic acid. First, the electrophoretic mobilities of the three silver nitrate positive compounds

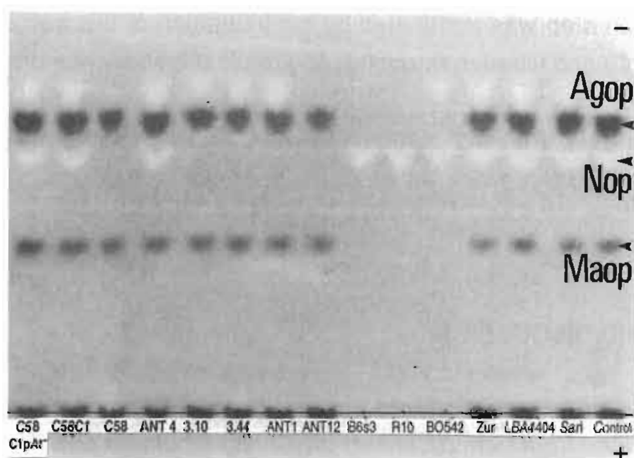

Figure 1: **Utilization of opines by various strains of *Agrobacterium.*** Strains ANT 4, 3.10, 3.44 and control strains were inoculated to the opine degradation assay medium. Samples of the medium were analyzed by high voltage paper electrophoresis at pH 1.9, five days after inoculation. In addition to strains decribed in Materials and Methods, other agrobacteria were either avirulent (Zur, LBA4404, Sari) or harbored a nopaline-type Ti plasmid (ANT1, ANT12), an octopine-type Ti plasmid (B6s3, R10) or an agropine-type Ti plasmid (Bo542). Legends are as follows: Agop, agropine; Nop, nopaline, Maop, mannopine. Symbols + and - indicate the polarity of the electrical field.

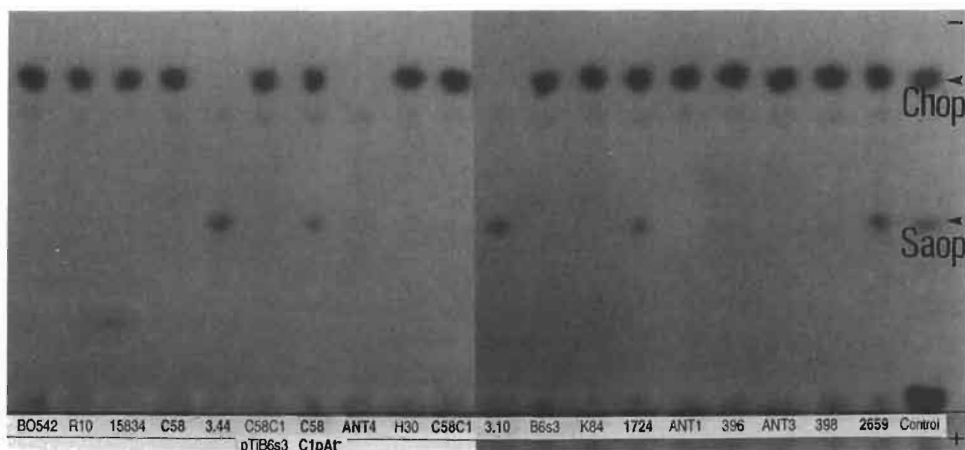

Figure 2: **Utilization of chrysopine and santhopine by various strains of *Agrobacterium*.** Large amounts of ANT 4-induced tumors were extracted as indicated in Materials and Methods. Strains ANT 4, 3.10, 3.44 and control strains were inoculated to the biological extract. Disappearance of opine-like molecules was assessed by high voltage paper electrophoresis at pH 1.9, three days after inoculation. In addition to strains decribed in Materials and Methods, other agrobacteria were either avirulent (H30, K84) or harbored a nopaline-type Ti plasmid (ANT1, ANT3), an octopine-type Ti plasmid (B6s3, C58C1pTiB6s3, R10) or an agropine-type Ti plasmid (Bo542, 396, 398). Additional control strains were *A. rhizogenes* isolates harboring an agropine-type Ri plasmid (15834), or a mikimopine Ri plasmid (1724), or a cucumopine-type Ri plasmid (2659). Legends are as follows: Chop, chrysopine; Saop, santhopine. Symbols + and - indicate the polarity of the electrical field.

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observed at two different pH were equivalent to those of the opines agropine, mannopine and agropinic acid. Also, the heat sensitivity and degradation patterns in diluted acid and alkali of agropine, mannopine and agropinic acid were similar to those of chrysopine, santhopine and chrysopine II (data not shown).

Biological properties of the three opine-like molecules. To decide whether the silver nitrate-positive molecules are indeed opines, tumor crude extracts were obtained and biologically purified as indicated in Materials and Methods. The biologically-purified crude extract was used to feed various *Agrobacterium* strains. Chrysopine was specifically degraded by the only inciting *Agrobacterium* strains, and should therefore be regarded as a *bona fide* opine (fig. 2). Chrysopine II was degraded by all strains utilizing mannopine, mannopinic and agropinic acids, and by strain ANT 4 (fig. 3). Remarkably, the two *Ficus* isolates 3.10 and 3.44 which induced chrysopine II synthesis in tumors,

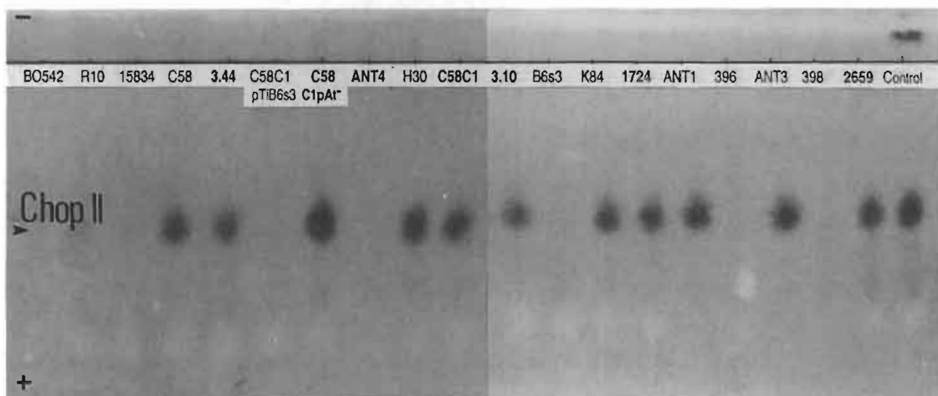

Figure 3: **Utilization of chrysopine II by various strains of *Agrobacterium*.** Large amounts of ANT 4-induced tumors were extracted and biologically depleted as indicated in Materials and Methods. Strains ANT 4, 3.10, 3.44 and control strains were inoculated to the biologically purified extract. Disappearance of opine-like molecules was assessed by high voltage paper electrophoresis at pH 2.8, five days after inoculation. In addition to strains decribed in Materials and Methods, other agrobacteria were either avirulent (H30, K84) or harbored a nopaline-type Ti plasmid (ANT1, ANT3), an octopine-type Ti plasmid (B6s3, C58C1pTiB6s3, R10) or an agropine-type Ti plasmid (Bo542). Additional control strains were *A. rhizogenes* isolates harboring an agropine-type Ri plasmid (15834), or a mikimopine Ri plasmid (1724), or a cucumopine-type Ri plasmid (2659). Legend is: Chop II, chrysopine II. Symbols + and - indicate the polarity of the electrical field.

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were not able to degrade this molecule (fig. 3). Santhopine was degraded by all strains of *Agrobacterium* except strains 3.10, 3.44, C58C1pAt⁻(the pTi- and pAt-free derivative of strain C58 used to deplete the tumor crude extract) and two *A. rhizogenes* strains: 1724 and 2659 (fig. 2). Very interestingly, strains C58 and C58C1 degraded santhopine. These results strongly suggest that santhopine degradation could be a function encoded by the large cryptic plasmid present in most of the *Agrobacterium* strains, including C58C1. Consistent with this, no large plasmid was seen in lysates obtained from strain 2659 which is also unable to degrade santhopine. If confirmed, santhopine degradation could be the first function associated with the large cryptic plasmid of *Agrobacterium*.

Analysis of the plasmid content of the new isolates. Plasmid content of the three isolates was investigated by the Eckhardt procedure. Strains ANT 4 and 3.44 contained at least three large plasmids, whereas strain 3.10 harbored

apparently only one large extra chromosomal replicon (data not shown). Though this technique only provides data on the number of plasmids contained in a given strain, analysis of the opine-related properties of these strains indicates that strain ANT 4 harbors a new type of Ti plasmid. Similarly, pTi 3.10 and pTi 3.44 probably belong to a new class of Ti plasmids, related to pTi ANT 4. Indeed, strains 3.10 and 3.44 induce tumors containing opines that are also detected in ANT 4-induced galls. However, strains 3.10 and 3.44 degrade neither santhopine, chrysopine II, nor leucinopine. Therefore, the pathogenic plasmids in these two later strains should not be identical to pTi ANT 4. Further studies on these plasmids are necessary to investigate their degree of relatedness and homology to other Ti plasmids.

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Characterization of different pathovars of *X. campestris*

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ABSTRACT

The possibility of different reisolates to synthesize the exopolysacharide with the variability of the monosacharide, pyruvate and acetate content was performed. It was shown that the synthesis of xanthan is geneticially determined. The chemical content of the xanthan from different reisolates didn't differ from the xanthan of the main strain.

INTRODUCTION

The taxonomic situation in the genus *Xanthomonas* at the generic, specific and subspecific levels has been explored by a variety of approaches (M.Star, 1981).
For some years there has been a trend towards reduction in the number of species of the plant pathogenic bacteria in general, particularly among the plant pathogenic representatives of Pseudomonas and in Xanthomonas. This trend is shown in the number of species of Xanthomonas has been reduced to five : *X.campestris* with which 102 nomenspecies (pathovar) are listed as synonyms, *X.ampelina*, *X.fragarie*, *X.albilineans* and *X.axonopodis* (A.Haward, 1979).
The phylopathogenic specifity is of great importance for the taxonomy of genus Xanthomonas. The most of the authors based on epydemilogical data received from the natural infections in the fields, proving the phytopathogenical specificity for the individual plant species (Klement, 1968).

The other authors have another opinon (Dye, 1958 and Shackleton, 1966) proved the possibility of variety of hosts and that the specificity of the host is not stable feature for Xanthomonas.
One of the products of *X.campestris* that is thought to contribure to pathogenisity is its extracellular polysaccharide, xanthan (or xanthan gum). Plugging of xylem plant tissue appears to be caused by synthessis of xanthan by colonizing *X.campestris* since it is found at the site of the lesion (Sutton, J.C. and Williams, P.H., 1970). Hewever, a direct link between the ability to synthesize xanthan and pathogenesis is not yet establised.
In this paper, we report the characterization of exopolysaccharide from different reisolates *X.campestris* pathovars: *vesicatoria*, *glicines*, *faseoli*, and corelation between EPS and virulence of this reisolates.

MATERIALS AND METHODS

Bacterial strains and reisolates.
We obtained 5 Xanthomonas campestris strains from Plant Protection Institute - Kostinbrod, BG :
X.campestris p.v. *vesicatoria* H 58
X.campestris p.v. *vesicatoria* H 42
X.campestris p.v. *vesicatoria* H 67
X.campestris p.v. glycines H 191
X.campestris p.v. glycines H 271

Strain *X.campestris* pv *phaseoli*-Tb was isolated previously in Lab-4 - Institute of Bioproducts - Plovdiv, BG. The reisolates from this strains were isolated from Plant Protection Institute-Kostinbrod (Bogatzevska, N. et al, 1992). The strains and the reisolates were maintained on YM agar (Jeanes, A. 1976). They were transfered once every 2 weeks to maintain good viability and stability for xanthan production. The cultivation media contains following components g/l: glucose- 40, KH PO- 6.8, NaNO- 2.0, MgSO .7, H O-0.2, citric acid- 2.0, CaCO- 0.2, H BO- 0.006, ZnO- 0.006, Fecl. 6H O-0,0024, the pH - 7.0 .
Culture conditions. Cultures were grown in 500 ml, Erlenmeyer flasks containning 100 ml of medium at 30 C for 96h. The flasks were shaken at 250 rpm on a New Brunswick rotary shaker. Inoculum was grown for 24h in YM-medium. After incubation 7 ml culture was used to inoculate the flasks with 100 ml medium.

Analytical Methods.

Xanthan was determined by precipitation with etanol after removing cells by the method of Jeanes et al (1976). Viscosities were measured with a Brookfield viscometer type DV-II, LVT, sp4, 30rpm at 25 C. Cell density was determined from optical density at 650 nm. Piruvic acid in xanthan was estimated by the method of Sloneker and Orentas (1962). Uronic acid content was measured by the carbazole method (Knutson and Jeanes, 1968). O-Acetyl was determined by the hydroxamic acid method (McComb, E.A, and Mc Cready, R.M., 1957).

Carbohydrate composition was determined in hydrolyzates by TLC-chromatographic separation of the components elution of individual sugars from the chromatogram and phenol - sulfuric acid analysis of the eluate (Sloneker and Jeanes, 1962).

RESULTS AND DISCUSSION

It is known a tittle of the nature of synthesized polysaccharide complex by *X.campestris* for the virulence of the bacteria. The presence of the exopolysaccharide is used characterizing *X.campestris* taxonomically. It is interesting to know if this genetically determined feature will be proved phenotipically through several generations reisolates and will correlate with the pathogenity of the strains. The obtained reisolates - R4 till fourth generation from strains of different pathovars were studied for their Xanthan formation. In table 1 are summerized the results till R4 from typical plant-hosts.

Phenotypic stability of the gene determing the synthesis of xanthan in different reisolates till generation R4 was shown. The strains of generation R2 of the studied strains showed higher activity of xanthan formation. Loosing of the ability to synthesized Phenotypic stability of the gene determing the synthesis of xanthan in different reisolates till generation R4 was shown. The strains of generation R2 of the studied strains showed higher activity of xanthan formation. Loosing of the ability to synthesized the xanthan was observed in strains H 58 for R3 and R4 and for strain H2 for R4 generation. At the same time very low vitality of these strains was observed. May be this is the reason of their impossibility to produce xanthan.The activity of producing xanthan in reisolates received from artifically infected non-typical plant-hosts was studied. The results are presented on table 2.

The obtained results show non-typical specificity of the different pathovars when they infected non-typical plant-hosts. Bazed on the results of the virulence of these strains and their polysaccharide synthesis could be supposed some correlation between these two taxonomic features. But for the three strain H42, H67, and T13 loosing the vittality of

Table 1. Biosynthesis of xanthan by reisolates till R4 isolated from typical plant-hosts

strain	parameters	R0	R1	R2	R3	R4
H42	viscosity (cps) xanthan (g/kg) biomass (g/kg)	11200 16.0 4.8	11940 16.5 2.5	12300 18.0 3.7	840 11.0 1.0	800 10.5 1.0
H67	viscosity (cps) xanthan (g/kg) biomass (g/kg)	1940 11.0 3.0	2420 15.0 4.0	11300 20.8 6.0	2720 15.6 4.2	2600 14.0 4.0
H58	viscosity (cps) xanthan (g/kg) biomass (g/kg)	9200 10.4 7.3	10500 14.0 7.5	7600 12.0 5.8	1960 8.0 1.2	830 4.5 1.0
H191	viscosity (cps) xanthan (g/kg) biomass (g/kg)	2230 12.4 3.0	2800 15.6 2.4	7700 19.3 4.1	5200 16.4 3.2	4450 16.0 3.5
H271	viscosity (cps) xanthan (g/kg) biomass (g/kg)	1460 11.0 3.0	1660 15.7 2.3	2070 15.4 3.6	2100 16.2 3.8	1980 15.7 4.2
T13	viscosity (cps) xanthan (g/kg) biomass (g/kg)	44600 11.4 3.0	5400 11.8 2.5	8200 13.1 3.5	6100 12.4 3.2	7680 12.8 3.6

Table 2. The biosynthesis of xanthan by reisolates, isolated from non-typical plant-host.

reisolates	H42		H67		H58		H191		H271		T13	
plant-hosts	visc. cps	EPS g/kg	visc cps.	EPS g/kg	visc. cps	EPS g/kg	visc. cps	EPS g/kg	visc. cps	EPS g/kg	visc. cps	EPS g/kg
Tomatoes R0							2270	12.4	1460	11.0	4660	11.4
R1							1360	9.0	7400	13.7	-	-
R2							1760	11.5	-	-	-	-
R3							3100	12.3	-	-	-	-
soyabean R0	11200	16.0	1940	11.0							4660	11.4
R1	2700	17.0	9350	14.3							1800	15.0
R2	9100	17.0	-	-							-	-
R3	5600	17.4	-	-							-	-
R0	11200	16.0			9020	10.3			1460	11.0		
R1	600	8.0			1800	13.7			1960	13.6		
R2	250	3.1			800	12.2			9180	14.4		
R3	0	0			-	-			2200	11.9		

Table 3. Monosaccharide content

strain	D-glu-cose	D-manose	D-glico-nicacid	D-gala-ctose	L-pam-nose	D-xy-lose	Pyru-vate	Ace-tate
H42	38.2	17.1	22.0	16.9	5.8	-	0.7	1.7
R4g	38.4	17.0	22.5	17.0	5.7	-	0.7	1.7
R3c	37.8	16.5	23.0	17.6	5.7	-	0.9	1.6
H67	37.4	16.9	22.3	17.6	4.2	1.6	2.4	1.8
R4g	37.6	17.5	22.6	17.1	4.3	0.9	2.6	2.4
R1c	36.7	17.2	23.0	17.3	5.1	0.7	1.8	1.6
H58	38.4	16.9	22.4	18.0	4.2	-	3.2	1.4
R4g	38.2	17.1	22.0	18.2	4.4	-	3.0	1.8
R2f	38.2	16.8	21.8	18.7	4.8	-	3.2	1.4
H191	40.8	38.4	20.8	-	-	-	4.2	2.5
R4c	41.8	38.0	20.6	-	-	-	4.0	2.8
R3g	40.6	38.6	20.8	-	-	-	4.2	2.5
H271	42.6	37.4	20.0	-	-	-	3.5	5.1
R4c	42.6	37.4	20.0	-	-	-	4.2	4.8
R3f	42.4	37.4	20.2	-	-	-	4.2	4.6
T13	41.6	28.4	27.6	-	2.4	-	3.6	1.4
R4f	41.4	28.2	27.6	-	2.8	-	3.4	1.4
R1c	40.8	27.8	28.6	-	2.8	-	3.6	1.4

generation R1 was observed. The relationship between these two taxonomic features could be established only after detail genetic study. But only one conclusion can be postulates: the biosynthesis of xanthan is obligatory for physiological and pathological active strains.
The monosaccharide content of xanthan synthesized by the studied strains and reisolates was proved to be the same in all cases (table 3). This content is constant independently of the change of plant hosts. We show the presence of galactose monomer in *X.campestris* p.v. *vesicatoria.* In the other studied strains this monomer was not detected.
Summerizing the results it can be concluded that the specificity of infection as plant-host-pathovar (p.v.) is not obligatory and consequently can't be used as a criteria for the determination of pv of *X.campestris*. The synthesized xanthan from different pv of *X.campestris* is typical and the variability in their monomer content are not useful as a criteria for differentiation of pathovars.

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Regulation of xanthan biosynthesis Some aspects of metabolite regulation of xanthan biosynthesis in *X. campestris*

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ABSTRACT

A correlation between the xanthan synthesis and the rate of cellular growth and assumption of glucose was studied.
Important factor the xanthan biosynthesis is the level of pyruvate in the cells.

INTRODUCTION

Xanthan is an extracellular heteropolisaccharide produced by *Xanthomonas campestris*. Xanthan gum has manifold application in the food and pharmaceutical industries and in oil field operations. Due to its commercial importance, the recovery and purification of xanthan from *X.campestris*. Fermentation broth has been subject of many studies.
The conventional batch process for xanthan gum manufacturing is expensive. The process may be improved by understanding better the metabolism of polysaccharide synthesis (Jarman *et al.*, 1984; Kennedy and Bradshaw, 1984).
During the fermentation a correlation between the xanthan synthesis and the rate of cell growth is studied as well the consumption of glucose. It was determined that in nitrogen controlled growth and the glucose is in an excess its conversion is not a criteria for xanthan biosynthesis. Such a controlling factor for the growth is the utilization of an excess of acidic groups from the polysaccharide (from acetate, pyruvate, glucoronic acid etc.).
So, instead of more pronounced energy effectivity of Citric acid cycle, the cells prefer the way of polysaccharide synthesis for energy conversion. This way is alternative for obligate aerobic organisms, when the Citric acid cycle is inhibited (Weis and Ollis, 1980).

The aim of the present work is the study of the level of pyruvate as a key metabolite for bacteria of genus Xanthomonas and its importance for xanthan biosynthesis.

MATERIALS AND METHODS

Microorganisms. We obtained two *Xanthomonas campestris* strains from Institue for Bioproducts - Plovdid: *Xanthomonas campestris* ITS-342 and *Xanthomonas campestris* pv.*vesicatoria* H41.

The strains were maintained on YM-agar. They were transferred once every two weeks to maintain good viability and stability for xanthan production.

The cultivation media contains following components, g/l: glucose- 40, KH PO- 6.8, NaNO- 2.0, MgSO. 7, H O- 0.2, citric acid- 2.0, CaCO- 0.2, H BO- 0.006, FeCL 0.6H O- 0.0024, destilate water to 1l. Glucose was sterilized separately. The solution was adjusted to pH- 7.0 before sterilisation.

Culture conditions. Cultures were grown in 500 ml Erlenmeyer flasks containing 100 ml of medium at 30 C for 72h. The flasks were shaken at 250 rpm on a New Brunswick rotary shaker.

Inoculum was grown for 24h in YM-medium. After incubation 7 ml culture was used to inoculate the flasks with 100 ml medium.

Analytical methods. The amount of xanthan accumulated in the growth medium was precipitated with two volumes ethanol and estimated by gravimetric method (Jeanes, Rogovin, Cadmus, 1976).

Cell density was determinated from optical density at 650 nm.

Viscosity was measured with a Broockfield viscosimeter type DV-II-LVT, spindale 4, 30rpm, at 25 C.

Glucose concentration in the cell-free supernatant was determined by the glucose-oxidase method, using Glucoanalyzer 2-Beckman.

The culture broth was diluted with NaCl solution to reduce the viscosity and was centifugated (16000 rpm for 30 min). The cells were washed once with 0.7% NaCl to remove residual polysaccharide. After centrifugation cells were used for determination of pyruvate.

Pyruvate in cells was determined by the methods described by Hmelevskii (Hmelevskii, 1985).

RESULTS AND DISCUSSION

In our previous study the key role of pyruvate for xanthan biosynthesis was shown (Iliev, et.al, 1990). The higher pool of pyruvate on the active strain and the lower production of the latter in the unactive strains show the alternativity for the biochemical pathways. CTA and the biosynthesis of xanthan for the generation of the energy consumption in the cells of *X.campestris.*

Some aspects of physiological control of xanthan biosynthesis exist in different strains of *X.campestris* independently of the genetic determination. Import influence on the xanthan production cause some primary metabolities from the CTA especially citric acid, fumaric acid and L-ketoglutaric acid (unpublished results). This could be explained in the terms of inhibition of CTA, when the biosynthesis of EPS is increaded. The mechanism of the inhibition probably is connected with equilibrium between ATP and HADH. The date are confirmed by the importance of pO for the xanthan production (O. Vashitz, 1990).

TABLE 1. The dependance of xanthan production from the initial quality of citric acid in the medium

citric acid (%)	pH	xanthan g/kg	biomass g/kg	Degree of conversion of glucose (%)
0	4.6	6.4	0.8	15
0.05	4.8	8.2	1.0	19
0.1	4.8	18.5	1.5	44
0.2	7.5	24.7	1.5	59
0.3	7.3	22.0	1.6	52
0.4	6.3	6.2	2.0	15
0.5	4.9	traces	2.5	-
0.6	4.6	traces	2.8	-

As it was shown the highest conversion of glucose into xanthan when the amount of citric acid is about 0.2-0.3%. The pH alterration also support the hypotesis that the whole amount of pyruvate and other acidic metabolities are neutralized through including in the molecule of the polymer. These results could be confirmed by the data showing on fig.1. In both cases the level of pyruvate in the cells increases.

But the pool is rather higher when citric acid present in the medium. The level of pyruvate at 24h of cultivation decreases dramatically. Probably this is due to the activating of the energetic processes in the cells (synthesis of ATP). At this time CTA isn't inhibited.

After this period the process is changed and the alternative pathways of xanthan biosynthesis occur and ATP inhibited the CTA. May be the citric amount not only buffer the fermentation medium but also enhances the stage of inhibition of CTA.

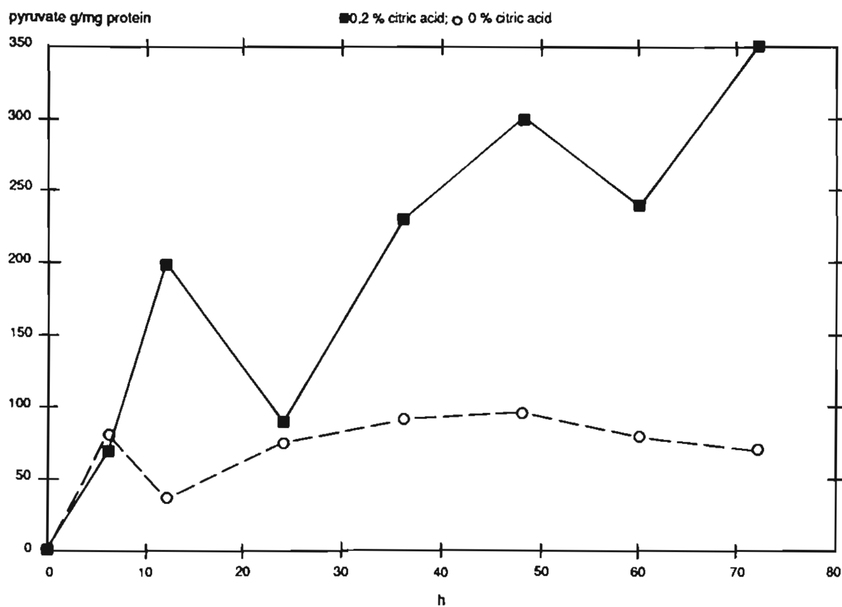

Fig.1. The dynamics of pyruvate pool in the cells of X.campestris ITS-342 in the presence and absence of citric acid in the medium

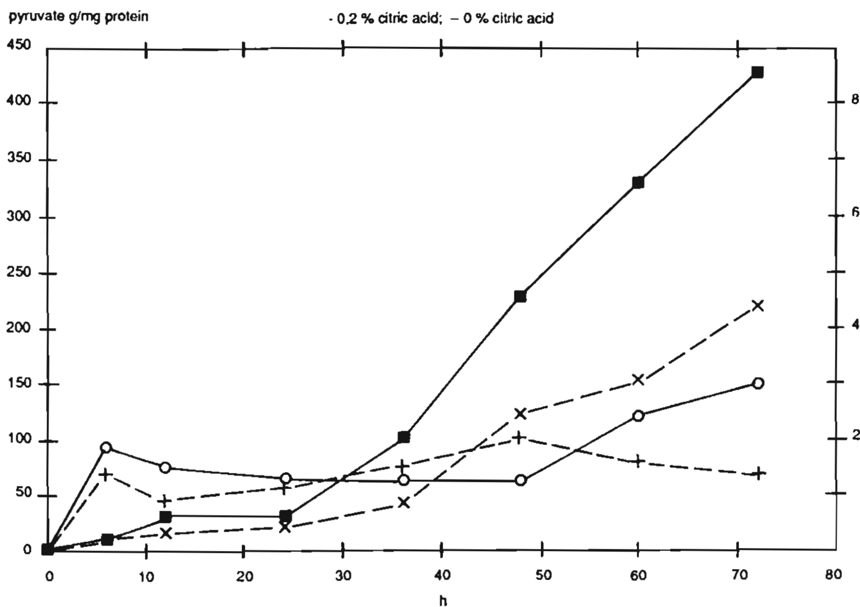

Fig.2. The dynamics of synthesis of the exopolysaccharide and pyruvate pool in X.campestris strain MX-9

The two fold decrease of pyruvate in the case (without citric acid) confirm our results. Our experiments were confirmed also with the data obtained with mutand MX-9 characterized with its resistance to bacitracin 500 g/ml and poorly producting xanthan (fig.2).

The absence of citric acid seem to be very important for the xanthan biosynthesis in this case too (fig.2). On the other hand once more is confirmed the leading role of pyruvate for the exopolysaccharide synthesis.
In conclusion is possible to say that mechanism determined by the pyruvate pool in the cells is apart of the complicated mechanisms of biochemical control. The energetic status of the active and unactive strains as well the activity of some main enzymes in CTA and their regulation will be the subject of the work performed in our laboratory in connection with patogeneticy of plant bacteria in the first stages of the diseases.

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Lipopolysaccharides of typical pectobacteria of genus *Erwinia*

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ABSTRACT

Westphal's method (1965) was used to isolate lipopolysaccharides (LPS) from six strains of *Erwinia carotovora* subsp. *carotovora*. LPS differ from one another by the qualitative and quantitative content of certain sugars. In contrast to monosaccharide composition of pectobacterial LPS, the fatty acids of lipid A are similar in different strains and highly specific. 3-oxytetradecanoic and dodecanoic fatty acids are basic in LPS lipid A of *E. carotovora*. Preliminary investigations demonstrated that 3-oxytetradecanoic and dodecanoic acids are the main fatty acids of LPS from *E. carotovora* ssp. atroseptica and E. carotovora ssp. *betavasculorum*. A method for the determination of the predominant fatty acids in LPS of bacteria without biopolymer isolation from the cell has been proposed.

KEYWORDS

Erwinia carotovora subsp., lipopolysaccharides, chemical composition, lipid A, fatty acids, systematics.

The members of *Erwinia carotovora*, so-called typical pectobacteria have weak or moderate affinity with other representatives of genus *Erwinia*. It was confirmed by the low level of DNA-DNA homology of these pathogens with members of genus Erwinia

(BRENNER et al., 1973), by their polyamines (GVOZDYAK et al., 1982), and the fatty acid composition of cell lipids. All these data show uncertainty of systematic position of these pathogens (LELLIOTT, DICKEY, 1984). Therefore, the members of *E.carotovora* need thorough study to determine their phylogenetic affinity both with the representatives of genus *Erwinia* and family of Enterobacteriaceae. The taxonomic significance of bacterial lipopolysaccharides (LPSs) is universally recognized (NICAIDO, 1970). The information on LPS from bacteria of genus *Erwinia* is limited (De BOER *et al.*, 1985; PREHM, 1985; RAY *et al.*, 1986) and still less is known about the lipid component of these LPSs (RAY *et al.*, 1986).

This work presents the characteristics of LPS from E. carotovora ssp. carotovora ATCC 15713, 48, 53, 213, 216 and 258. The strains differed from each other by the monosaccharide composition of intact cells. For the determination of prevalent fatty acids in the composition of lipid A we have used *E. carotovora* ssp. atroseptica NCPPB 549, *E.carotovora* ssp. *betavasculorum* NCPOPB 2795 and *E. carnegieana* NCPPB 671. At the moment of examination all pectobacteria had high pectolytic activity.

The cultivation of bacteria, their preparation to test, LPS extraction, sedimentation and purification as well as the determination of their chemical composition were carried out as described earlier (MOROS et al., 1989). For the determination of fatty acid composition of lipid A the biopolymers were subjected to methanolysis with 5% acetyl chloride in methanol for 4 hours at 100 C.

The extraction of methyl-ethers of fatty acids, their separation and identification were carried out as described earlier (MOROS et al., 1989).

For the determination of the prevalent fatty acids in the composition of pectobacterial LPS without isolation of biopolymers from cells, the extractable and bound lipids were extracted according to the method of Blight and Dyer (1959). Intact cells, phospholipid - free cells as well as phospolipid fraction and LPS of these bacteria were subjected to methanolisis.

The yield of LPS at their extraction from cells with hot phenol-water mixture was low (from 1.4 to 2.3 % from cell weight). Carbohydrate made up from 30.1 to 36.7% from biopolymer dry weight. In the LPS composition the admixtures of nucleic acids (0.1-0.5 % from preparation weight) and a negligable protein amount (0.4-2.2% from cell weight) were found. The monosaccharide composition of biopolymers of *E. carotovora* ssp. *carotovora* was heterogenous. Heptose, glucose, galactose, mannose, arabinose and glucosamine were found in the LPS composition of all strains. LPSs of the agents studied differed from each other by the presence of rhamnose or fucose and lipo[illegible]c sugars (Table1).

Table1. Monosaccharide composition of LPSs from *E*. carotovora ssp. carotovora

Monosaccharides and aminosugars	Content of monosaccharides (in percentage from total square of peaks) and ;aminosugars (mkg per mg of preparation) in LPS of strains					
	15713	258	53	48	216	113
Non-identified 1	-	-	5,6	-	-	-
Non-identified 2	-	7,8	-	-	-	-
Non-identified 3	7,7	8,2	14,5	-	-	-
Rhamnose	15,5	24,4	-	-	21,2	13,4
Fucose	-	20,0	37,8	24,6	-	-
Arabinose	3,6	2,0	4,4	7,1	7,0	3,7
Xylose	-	1,9	1,4	1,0	-	-
Mannose	9,9	1,5	14,3	13,3	3,2	10,7
Galactose	20,1	17,7	2,9	14,6	16,5	26,9
Glucose	28,7	8,7	10,3	23,7	28,7	30,0
Heptose	14,5	7,8	8,8	16,7	23,4	15,2
Glucosamine	30,8	30,8	30,8	28,7	26,5	28,7

In LPSs of strains 48, 113, 216 no lipophylic sugars were found. In strain ATCC 15713 we one lipophylic sugar, and in LPS of strains 53 and 258 - by two such sugars out of which one was similar to that in strain 15713. Two other lipophylic sugars in LPS of strains 53 and 258 differed by their mobilities. It is notable that LPSs of strains studied have high content of lipophylic sugars. In strain 258 they made up 16 % from total monosaccharides, in strain 53, 20%. In biopolymers of all strains of *E.carotovora* ssp. carotovora the manose has been discovered though its content in two out of six strains was low. The other authors found the rare mannose in LPS composition of *E. carotovora* (De BOER, 1988).

In contrast to the monosaccharide composition of LPS from *E.carotovora* ssp. *carotovora* ssp. *carotovora* the fatty acid composition of lipid component of these biopolymers is similar. The 3-oxytetradecanoic acid made up 40-50 % dodecanoic - 30-35 %, tetradecanoic acid - 5-7 % from total fatty acid of lipid A. The variation of these values depends on the purification level of biopolymers from phospholipids. Besides, in the composition of LPS lipid A of typical pectobacteria the hexadecanoic, hexadecanoic and in some cases octadecenoic acids have been found.

Their content greatly decreased in a course of LPS purification from phospholipids. The amount of 3-oxytetradecanoic and dodecanoic acids changed insignificantly. The results obtained permitted to conclude that 3-oxytetradecanoic and dodecanoic acids are constitutive fatty acids of LPS lipid A of *E.carotovora* ssp. *carotovora.* The other fatty acids found in spectrum, are a result of insuffient purification of biopolymers from cell lipids.

The preliminary experiments showed that 3-oxytetradecanoic and dodecanoic acids are also the main fatty acids of LPS lipid A of *E. carotovora* ssp. *atroseptica* NCPPB 549 and E. *carotovora* and *E. carotovora* ssp. *betavasculorum*, i.e. fatty acid composition of LPS lipid A of *E. carotovora* is specific on the level of species and differs from LPS lipid A of E. amylovora and *E. chrysanthemi.* As lipid A is the most conservative part of LPS its fatty acid composition can serve as an important taxonomic marker of these pathogens.

However, the isolation of LPSs and studing their chemical composition is a difficult and protracted procedure. Taking into consideration the significance of the test we worked out the method of the determination of the prevalent fatty acids of lipid A without isolation of LPS from cell. The point of the method is the release of cell from lipids which are not components of LPS. These lipids, in contrast to lipid A, are more easily extractable from cell strutures. The spectra of fatty acids of intact cells of E. carotovora ssp. carotovora 15713, phospholipid - free cells LPS of this strain extracted with phenol and fractions of lipids extracted has been stading . Comparing fatty acid composition of intact cells and total fraction of the lipids it is seen that both saturated and non - saturated fatty acids having 16 and 18 carbon atoms are easily extractable from cell. Only 3-oxytetradecanoic and dodecanoic fatty acids have not been observed in the composition of lipids extracted. Tetradecanoic acid has been found in fraction of lipids extractable as well as in phospholipid-free cells. In the composition these cells, the fatty acids having 16 and 18 carbon atoms have been also discovered, but their content was low. The 3-oxytetradecanoic and dodecanoic acids prevailed in phospholipid-free cells as in LPS of this strain. The method was tested on strains the fatty acid composition of lipid A of which is known.

Fatty acid composition of cell lipids of *E.carnegieana* NCPPB 671 differed from that of *E.carotovora* and was similar to the composition of *E.amylovora*. In cells of the agents freed from phospholipids the ratio of 3-oxytetradecanoic, dodecanoic and tetradecanoic acids corresponded to that of *E.amylovora*. In the composition of lipid A of LPS of *E.amylovora*, *E.chrysanthemi* and *E.carotovora* the amounts of dodecanoic and tetradecanoic acids varied. Thus in LPS of *E.carotovora* the dodecanoic acid prevailed, in lipid A of LPS from *E.chrysanthemi* dodecanoic acid was absent while in lipid A of LPS of *E.amylovora* the amounts of dodecanoic and tetradecanoic acids were almost similar (RAY *et.al.*, 1986).

Analysing the results we can conclude that *E. carotovora* and *E. chrysanthemi* can not belong to the genus *Erwinia* since they differ by fatty acid composition of lipid A from each other and from *E. amylovora*, type species for genus Erwinia.

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Variations among the strains of *Xanthomonas* isolated from citrus in the sensitivity to antibiotics

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ABSTRACT

65 strains representative of the different pathotypes of *X. campestris* pv. *citri*, the agent of Citrus Bacterial Canker Disease (CBCD), and six strains of *Xanthomonas* responsible for Citrus Bacterial Spot Disease (CBSD) were studied for their *in vitro* sensitivity to 63 antibiotics using a disc test.

All the strains were similarly resistant to four antibiotics, and a variability in the response was observed with the others. Hierarchical cluster analysis of the diameters of zone on inhibition revealed differences between strains of Mascareignes was distinguished. These strains shared a higher resistance to penicillins and cephalosporins. Strains of pathotypes B and D were clearly related, except one strain of the B type. Strains from CBS and strains of the pathotype C exhibited some variability.

These data allowed the selection of an appropriate pool of antibiotics to compose a semi-selective medium for the detection of *Xanthomonas* strains of CBCD and CBSD.

KEYWORDS

Xanthomonas campestris, Citrus bacterial canker disease, Citrus bacterial spot disease, pathotypes, epidemiological marker, identification.

INTRODUCTION

Strains of *Xanthomonas campestris* are responsible of two bacterial foliar diseases on citrus. *X.c.* pv. *citri* is the causal agent of Citrus bacterial canker disease (CBCD) which is widespread in the tropical area. Four variants of this pathogen (called pathotypes A, B, C and D) were initially described according to their host range and their geographical origin. Recently, in 1984, a new foliar disease, named Citrus bacterial spot disease, was found in Florida.

The causal agent is known as *X.c.* pv. *citri* pathotype E or *X.c.* pv. *citrumelo* (GRAHAM & GOTTWALD, 1991 ; STALL & CIVEROLO, 1991).

Different laboratory techniques permitted the characterization of each type and confirmed the differences between these pathotypes (Biochemical tests, serological tests using polyclonal and monoclonal antibodies, RFLP analyses...). However no difference related to pathogenic specification or geographical origin was detected among strains belonging to a same type (ALVAREZ *et al.*, 1991 ; HARTUNG & CIVEROLO, 1989 ; VERNIERE *et al.*, 1991).

We examined the variations in the sensitivity to antibiotics of representative strains of *X. campestris* isolated from citrus trying to improve the similarities or differences between the pathotypes or strains belonging to a same type.

MATERIALS AND METHODS

Sixty five strains of CBCD (53 strains of pathotype A originating from 23 countries, 8 strains of pathotype B, 3 strains of pathotype C and the single D type strain) and six strains of CBSD obtained from freeze-dried cultures were tested.

The sensitivity to 63 antibiotics was carried out using an *in vitro* antimicrobic diffusion test. Six discs (Diagnostics Pasteur - 92430 Marnes La Coquette - France) were applied on plates containing recently poured YPDA medium (yeast extract 7 g, peptone 7 g, dextrose 7 g, agar 15 g) using a distributor. After two days incubation at 28°c, the diameter of inhibition was measured. Each combination was repeated twice.

Numerical analysis of phenotypic features : the characters states were quantitative and analysis was performed using the average diameter as the clustering criterion. Hierarchical cluster analysis was carried out using the minimization of intraclass variance.

RESULTS

All the strains of CBCD and the six strains of CBSD were resistant to Trimethoprim, Clindamycin, Lincomycin and Metronidazole.

Four strains (CFBP2901 / B type, CFBP2910 / E type, CFBP2906 / C type and CFBP2857 / A type) were strongly differentiated from the other pathotypes (Figure 1). CFBP2901 was more sensitive, and a larger diameter using Penicillins and Cephalosporins was observed than those obtained with the other B types (Table 1).

Mascareignes (JJ9-3 from Mauritius and JJ10-3 from Rodriguez) showed a profile similar to that of the other type A strains.

Table 1 : variations of *X. campestris* isolated from citrus in the sensitivity to penicillins and cephalosporins.

Pathotypes		type A (3)	type A Masca (4)	type A Mauritius	types B + D(5)	type B	type C	type E (CBS)
number of strains tested		(x 37)	(x 15)	(JJ9-3)	(x 8)	(CFBP 2901)	(x 3)	(x 6)
PENICILLINS								
ampicillin	a(1)	9,1	6	6	27,6	51	6	16,5
	v(2)	35,8	0		6,1		0	58
amoxycillin	a	9,1	6	6	42,3	55	8,5	17,5
	v	50,5	0		6,7		18,8	115
carbenicillin	a	31,8	6,7	36	41,5	55	12,5	40,2
	v	62,9	2,35		3,8		127	12,2
mezlocillin	a	28,2	15,3	36,5	29,9	40	20,3	30,5
	v	28,3	26,6		7,0		7,6	11,1
ticarcillin	a	30,1	6,06	6	43,9	59	12,5	40,4
	v	54,4	0,06		6,0		108	18,0
CEPHALOSPORINS								
cephalexin / 1	a	6	6	6	6	24	8,0	6,6
	v	0	0		0		12,0	2,6
cefazolin / 1	a	6,07	6,03	6	6	34	7,6	7,6
	v	0,09	0,02		0		8,3	7,0
cefamandole/2	a	19,3	7,4	25	20,5	41	13,0	23,6
	v	30,5	5,5		12,4		57,3	18,8
cefuroxim / 2	a	11,4	6,9	13	19,1	44	12,8	19,0
	v	35,2	3,6		9,03		80,6	66,7
cefotiam / 3	a	8,1	6,1	6	21,4	45	11,2	17
	v	27,2	0,07		6,4		80,1	79,9
cefsulodin	a	29,2	6,9	34,5	35,9	51	17,7	36,2
	v	41,4	9,5		5,7		88,6	13,4

(1) average (mm) of the diameters of inhibition - the diameter of the disc is 6 mm.
(2) variance of the diameters of inhibition.
(3) pathotype A strains, strains from Mascareignes not included.
(4) strains from Mascareignes, strain JJ9-3 not included.
(5) pathotypes B and D, strain CFBP2901 not included.

Hierarchical cluster analysis revealed a hightly separated group (index of relative distance ID = 0,818) which included fourteen isolates (on sixteen) from the Mascareignes archipelago (Réunion, Mauritius and Rodriguez islands) and one strain from Oman (JF90-5) (Figure 1). These strains were distinguished from the other A type strains according to the higher resistance exhibited with Carbenicillin, Ticarcillin, Cefamandole and Cesulodin (Table 1).

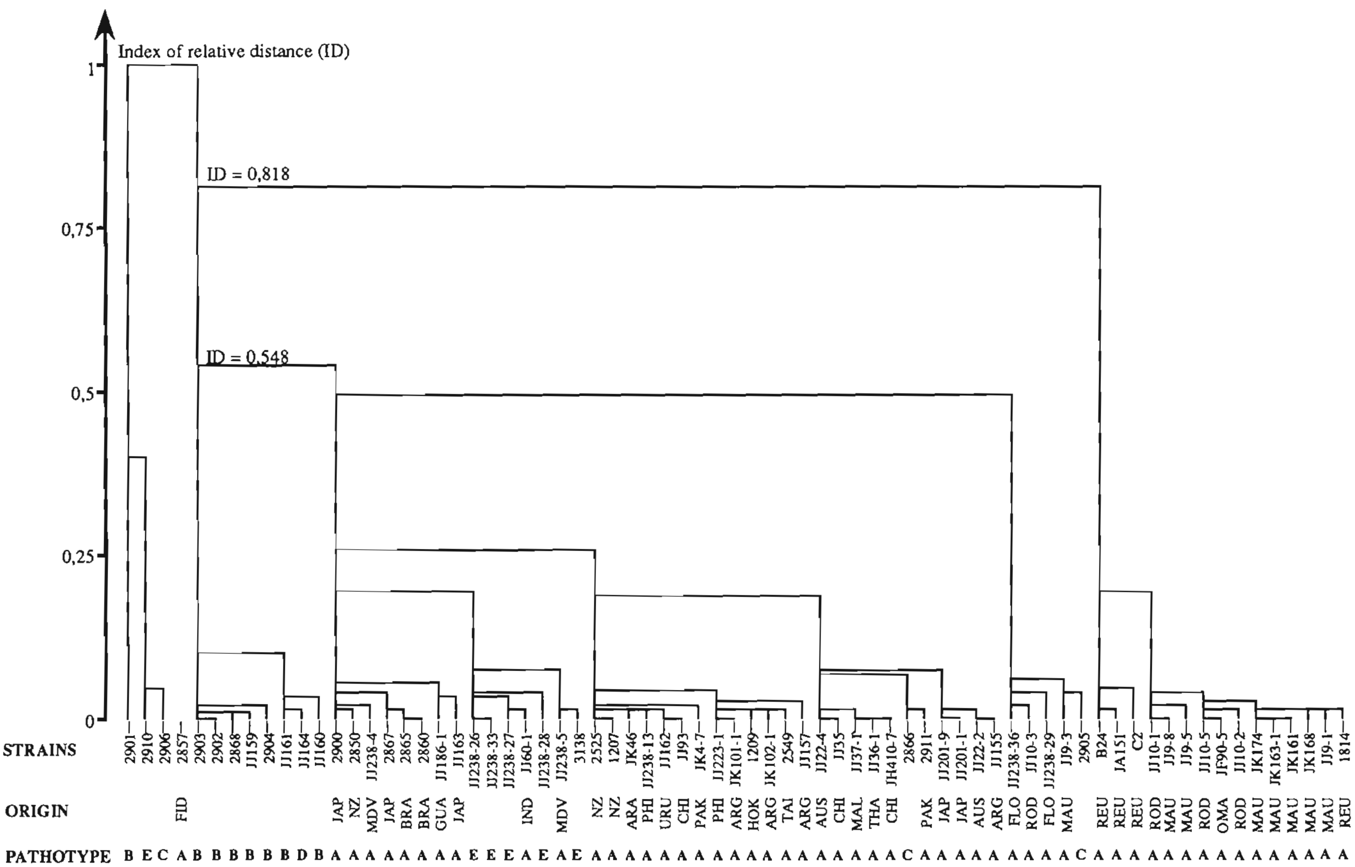

FIGURE 1 : Dendrogramm of relative distance grouped by minimization of intraclass variance showing the clustering among 71 strains of *X. campestris* from citrus based on their sensitivity to 59 antibiotics.

All the B type strains tested (except CFBP2901) and the single D type strains shared an isolated cluster (ID = 0,548). They were differentiated from the type A by their sensitivity to ampicillin, amoxicillin and cefotiam (Table 1).

Five strains of CBSD of six were clearly related and the three C pathotype strains tested were not clustered.

DISCUSSION

Clustering criterion used in this study separated more according to the geographical origin rather than the pathotype classification. Pathotypes A strains from Mascareignes (a tiny archipelago in Indian ocean) were strongly isolated. They are characterized by higher resistance to some Cephalosporins and some Penicillins. Two strains tested from Mauritius and Rodriguez islands had the same profile that the other type A strains. Introduction of plant materials from neighbouring countries could explain the presence of this little variation. The existence of such resistance in strains of *X.c.* pv. *citri* originating from Mascareignes is misunderstood. It could be used as markers for epidemiological studies.

Strains from B and D types were clearly related and B strain CFBP2901 exhibited a different profile as shown by different techniques (ALVAREZ *et al.*, 1991 ; VERNIERE *et al.*, 1991). No clustering appeared with C strains.

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Evaluation of metabolic fingerprinting as a tool to identify xanthomonads associated with two bacterial diseases of citrus

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ABSTRACT Metabolic profiles of 148 strains of *Xanthomonas campestris* pv. *citri* originating from 24 countries and associated with various forms of citrus bacterial canker disease (CBCD), and of 43 strains of *X. campestris* associated with citrus bacterial spot disease (CBSD) in Florida were obtained using the Biolog system. Metabolic profiles were used to attempt identification using the Microlog 2N database. Thirty three % of the tests done with *X. campestris*/CBS and only 7 % of the tests done with *X. c.* pv. *citri* gave correct identifications when the commercial database was used alone. When supplemented with data of 54 strains of *X. c.* pv. *citri* and of 43 strains of *X. campestris* associated with CBSD, the percentage of correct identifications was respectively 70 % for *X. c.* pv. *citri* and 64 % for *X. campestris*/CBS. Thus, it is recommended that users supplement the commercial database with additional data prior to using the program for identification purpose. The oxidation of tween 40 can be used as a convenient marker for differentiating strains associated with CBCD and CBSD. The oxidation of L - fucose, D - galactose, and alaninamide can be used as markers for differentiating strains associated with Asiatic citrus canker (CBCD - A), cancrosis B (CBCD - B), and Mexican lime canker (CBCD - C). These results confirmed the separation of these bacteria into different subgroups. A single strain associated with bacteriosis of Mexican lime in Mexico (CBCD - D) was closely similar to type B strains.

KEYWORDS :*Xanthomonas* - *citri* - Citrus Bacterial Canker Disease - Pathotype - Citrus Bacterial Spot Disease - Identification - Metabolic fingerprinting - Biolog - oxidation - carbon source.

Citrus bacterial canker disease (CBCD) is endemic in many producing countries (KOIZUMI, 1985). Several forms of CBCD have been described. Their differentiation is based on host range, geographic origin, biochemical tests, phage typing, serology using polyclonal or monoclonal antibodies, genomic and/or plasmid DNA fingerprinting, and RFLP analyses (CIVEROLO, 1984 ;

PRUVOST *et al.*, 1992 ; VERNIERE *et al.*, 1992). Existence of *X. c.* pv. *citri* pathotypes has been confirmed with all of the above mentioned techniques, although strains associated with CBCD - B (a disease restricted to *Citrus limon* in Argentina, Uruguay and presumably Paraguay) have a high similarity to the one available strain currently believed to be associated with CBCD - D (a disease restricted to *Citrus aurantifolia* in Mexico).

Citrus bacterial spot disease (CBSD), another disease of citrus caused by a bacterium identified as *Xanthomonas campestris* was discovered in Florida citrus nurseries in 1984 (SCHOULTIES *et al.*, 1985). This causal agent produces leaf and twig lesions which differ from those caused by *X. c.* pv. *citri*, and CBSD is of minor importance compared to Asiatic citrus canker, the most virulent form of CBCD. Two main subgroups of strains associated with CBSD (CBS - A and CBS - B), differing strongly in aggressiveness, occur (HARTUNG & CIVEROLO, 1991). Several laboratory techniques, which allow a good classification of strains into these subgroups, are available (HARTUNG & CIVEROLO, 1991 ; STALL & CIVEROLO, 1991). The taxonomic position of the strains associated with CBSD is still unresolved (STALL & CIVEROLO, 1991).

Recently, a technique based on metabolic fingerprinting was developed by Biolog Inc. (HAYWARD, CA - USA) to identify Gram - negative bacteria. This rapid technique is based on the ability of strains to differentially oxidize 95 carbon sources. Tetrazolium violet is used as a redox dye to visualize the increased respiration of bacteria while oxidizing a carbon source (BOCHNER, 1989). Identification can generally be performed within 24 hours. Metabolic profile of the test bacterium is compared to that of known bacteria whose profile is entered into a database. The Biolog system allowed a reliable identification at the species level of *Legionella* spp. (MAUCHLINE & KEEVIL, 1991). Among plant pathogens, this technique was quite useful for demonstrating metabolic variation among strains of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* from several countries (GRIFFIN *et al.*, 1991).

Since the design of a Biolog assay is much less laborious than other available techniques, it was evaluated as a diagnostic tool to differentially identify xanthomonads associated with bacterial diseases of citrus.

MATERIAL & METHODS One hundred forty eight strains of *Xanthomonas campestris* pv. *citri* originating from 24 countries (Argentina, Brazil, China, Christmas Island, Guam, Honk Kong, India, Japan, Korea, Malaysia, Maldives Islands, Mauritius Island, Mexico, New Zealand, Pakistan, Philippines, Reunion Island, Rodriguez Island, Taiwan, Thailand, Thursday Island, Uruguay, U. S. A. [Florida], and Yemen) and associated with various forms of CBCD, and of 43 strains of *X. campestris* associated with CBSD in Florida were used in this study.

Cultures obtained from freeze - dried ampoules or from silica gel were streaked on NGA (nutrient agar 23 g, glucose 10 g, distilled water 1000 ml, pH 6.8) plates to check purity. One colony was then subcultured on TSA medium (bactotryptone 15 g, bactosoytone 5 g, NaCl 5 g, bactoagar

15 g, distilled water 1000 ml, pH 7.3). TSA plates were incubated at 28°C for 24 hours. Since strains associated with CBCD - B/C/D are more sensitive to NaCl than strains associated with CBCD - A (VERNIERE *et al.*, 1991), a slightly modified procedure was used for these strains. They were grown on a modified TSA medium without NaCl. Biolog GN plates were generally used as recommended by the manufacturer. A slightly modified procedure was used for strains associated with CBCD - B/C/D. In this case, bacterial suspensions in sterile distilled water were used to inoculate the plates. Since the B/C/D strains have a slower growth than strains associated with CBCD - A and CBSD, oxidation of the carbon sources was delayed. Thus, plates were read after incubation for 48 hours at 28°C. Identifications were performed using the Microlog 2N software as recommended by the manufacturer. Attempts to identify strains were first done using the Microlog 2N database alone. Subsequently, the data from 40 strains of *X. c.* pv. *citri* associated with CBCD - A (isolated in various countries from various host species), 8 strains associated with CBCD - B (isolated in Argentina and Uruguay from lemon), 6 strains associated with CBCD - C (isolated in Brazil from mexican lime), and 43 strains of *X. campestris* isolated in Florida and associated with CBSD were used to supplement the commercial database and the software was used again for identification.

RESULTS and DISCUSSION Obtained fingerprints were nearly, but not completely reproducible. Problems in reproducibility were identical to those previously reported (HARTUNG & CIVEROLO, 1991). Atypical reactions occurred in A5 well (which contains tween 40) in the microplates for some of the strains of *X. c.* pv. *citri*. The reactions in this well were almost always recorded as positive, although there was no apparent purple color. This phenomenon was sometimes recorded for A6 well (which contains tween 80) too.This was explained by the formation of a precipitate composed of white material. This obviously was not a positive reaction. Manual correcting of the fingerprint in the software was performed prior attempting identification. However, a typical positive reaction was observed in these two wells for almost all strains associated with CBSD.

Carbon sources oxidized by all strains were : glycogen, cellobiose, D - fructose, α - D - glucose, maltose, D - mannose, sucrose, mono - methylsuccinate, α - ketoglutaric acid, succinic acid, bromosuccinic acid, and glycyl - L - glutamic acid.

Carbon sources oxidized by none of the strains were : N - acetyl - D - galactosamine, adonitol, D - arabitol, i - erythritol, m - inositol, β - methylglucoside, L - rhamnose, xylitol, formic acid, D - galactonic acid lactone, D - galacturonic acid, D - gluconic acid, D - glucosaminic acid, D - glucuronic acid, γ - hydroxybutyric acid, p - hydroxyphenylacetic acid, itaconic acid, α ketovaleric acid, quinic acid, sebacic acid, glucuronamide, L - histidine, L - ornithine, L - phenylalanine, L - pyroglutamic acid, D.L - carnitine, γ - aminobutyric acid, thymidine, phenylethylamine, putrescine, and 2 - aminoethanol.

Variable reactions were recorded for the following substrates : α - cyclodextrin, dextrin, tween 40, tween 80, N - acetyl - D - glucosamine, L - arabinose, L - fucose, D - galactose,

gentiobiose, α - lactose, lactulose, D - mannitol, D- melibiose, psicose, D - raffinose, D - sorbitol, D - trehalose, turanose, methylpyruvate, acetic acid, cis - aconitic acid, citric acid, α - hydroxybutyric acid, β - hydroxybutyric acid, α - ketobutyric acid, D. L - lactic acid, malonic acid, propionic acid, D - saccharic acid, succinamic acid, alaninamide, D - alanine, L - alanine, L - alanyl - glycine, L - asparagine, L - aspartic acid, L - glutamic acid, glycyl - L - aspartic acid, hydroxy L - proline, L - leucine, L - proline, D - serine, L - serine, L - threonine, urocanic acid, inosine, uridine, 2 - aminoethanol, glycerol, D. L - α - glycerolphosphate, glucose - 1 - phosphate, and glucose - 6 - phosphate.

Tween 40 can be regarded as a nearly differential marker for identifying strains associated with CBCD and CBSD, since no strain of *X. c.* pv. *citri* oxidized this carbon source and 91 % of the strains of *X. campestris*/CBS oxidized it. Metabolic fingerprinting is useful for differentiating strains of *X. c.* pv. *citri* associated with different forms of CBCD (Table 1). A limiting factor is that the number of strains associated with CBCD - B, - C, -D available in the world is low. However, alaninamide, L - fucose, and D - galactose still can be used as convenient markers.

TABLE 1 : Differential oxidation of 3 carbon sources by strains of *X. c.* pv. *citri* associated with various forms of CBCD.

L - fucose	D - galactose	alaninamide	form of CBCD
+	+	+	A[a]
+	-	+	A[b]
-	+	+	A[c]
-	-	+	B/D
+	+	-	C

[a]77.8 % of strains associated with CBCD - A shared this profile.

[b]3.8 % of strains associated with CBCD - A shared this profile.

[c]18.4 % of strains associated with CBCD - A shared this profile.

No carbon source allowed a clear - cut differentiation of members of *X. campestris* groups CBS - A and CBS - B. D - raffinose, glycil - L - aspartic acid, and D. L - a - glycerolphosphate were nearly differential.

When using the Microlog database alone, only 7 % of the strains of *X. c.* pv. *citri* and 33 % of the strains of *X. campestris*/CBS were correctly identified.

Identification attempts were done again after the database was supplemented with metabolic profiles of 54 strains of *X. c.* pv. *citri* (40 associated with CBCD - A, 8 associated with CBCD - B, and 6 associated with CBCD - C) and of 43 strains of *X. campestris*/CBS (24 belonging to group A and 19 to group B). Identification results are summarized in Table 2. Sixty seven, 94, and 100 % of the strains of *X. c.* pv. *citri* respectively associated with CBCD - A, - B, and -C were correctly identified. No strain of pv. *citri* was identified as *X. campestris*/CBS. Strains belonging to the CBS- A group were

Table 2 : Identifications of strains of *X. c.* pv. *citri* associated with different forms of CBCD and of *X. campestris* associated with CBSD as performed by the Microlog 2N software's database supplemented with data from 54 strains of *X. c.* pv. *citri* and 43 strains of *X. campestris* associated with CBSD.

Identification[a]	CBCD - A n = 133	CBCD - B n = 8	CBCD - C n = 6	CBCD - D n = 1	CBSD - A n = 24	CBSD - B n = 19
X. c. pv. *citri* CBCD - A (Sim ≥ 0.750)	**53,2**[b]	0	0	0	0	0
X. c. pv. *citri* CBCD - A (0.500≤ Sim< 0.750)	**13,9**	0	0	0	4,2	0
X. c. pv. *citri* CBCD - B (Sim ≥ 0.750)	0	**75,0**	0	0	0	0
X. c. pv. *citri* CBCD - B (0.500≤ Sim< 0.750)	0,2	**18,8**	0	**100**	0	0
X. c. pv. *citri* CBCD - C (Sim ≥ 0.750)	0	0	**45,8**	0	0	0
X. c. pv. *citri* CBCD - C (0.500≤ Sim< 0.750)	0,2	0	**54,2**	0	0	0
X. campestris CBSD - A (Sim ≥ 0.750)	0	0	0	0	**25,0**	2,6
X. campestris CBSD - A (0.500≤ Sim< 0.750)	0	0	0	0	**24,0**	10,5
X. campestris CBSD - B (Sim ≥ 0.750)	0	0	0	0	5,2	**43,4**
X. campestris CBSD - B (0.500≤ Sim< 0.750)	0	0	0	0	9,4	**7,9**
X. c. pv. *vitians* sbgp. A (Sim ≥ 0.500)	0,2	0	0	0	0	0
X. c. pv. *dieffenbachiae* sbgp. B (Sim ≥ 0.500)	10,3	0	0	0	7,3	3,9
X. c. pv. *alfafae* (Sim ≥ 0.500)	0,2	0	0	0	0	0
X. c. pv. *vesicatoria* (Sim ≥ 0.500)	1,1	0	0	0	0	0
X. c. pv. *manihotis* (Sim ≥ 0.500)	5,5	0	0	0	0	0
X. c. pv. *ti*[c] (Sim ≥ 0.500)	0,2	0	0	0	0	0
X. c. pv. *turf*[d] (Sim ≥ 0.500)	0	0	0	0	0	6,6
Genus identification	7,9	0	0	0	20,8	18,4
Poor identification	5,8	6,3	0	0	4,2	1,3
No identification	1,3	0	0	0	0	2,6

[a]Nomenclature is as given by the Microlog 2N software and may not reach international standards for naming pathovars of phytopathogenic bacteria. /[b]Data are expressed in %. /[c]A *X. campestris* pathogenic on ti (*Cordyline terminalis*). /[d]A *X. campestris* pathogenic on turf grass.

identified as such in 49 % of the tests and were identified as CBS - B in 15 % of the tests. Similar results were obtained for strains belonging to the CBS - B group (51 % of the tests gave correct identifications ; identifications as CBS - A were recorded for 13 % of the tests). Profiles that belonged to CBS - A strains were identified as *X. c.* pv. *citri* in 4 % of the tests. In the case of misidentifications, the best matches were always members of *X. campestris*. Since the rate of correct identifications was increased by supplementing the commercial database with additional data, it is recommended that users do so prior to using the program for an identification purpose. We conclude that metabolic fingerprinting is a useful technique, but that the accuracy of an identification based only on this technique is questionable. Thus, it must be combined with other available methods to distinguish pathovars of *X. campestris*. However, pathovars of *X. campestris* are by definition impossible to distinguish by physiological tests. In this context, the results presented in this study are very good.

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Cellular fatty acid composition of pectobacteria as evidence of their separate position from each other and from the other species of the genus *Erwinia*

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ABSTRACT

Erwinia carotovora (E. carotovora ssp. atroseptica, E. carotovora ssp. betavasculorum, E. carotovora ssp. carotovora) and E. chrysanthemi differ among themselves by cellular fatty acid composition as well as from *E. amylovora* and *Escherichia coli* and other species of genus *Erwinia*.

KEYWORDS

Enterobacteriaceae, *Erwinia* ssp., fatty acids.

INTRODUCTION

The data on the number of really existing species of genus *Erwinia* and their systematic position are very contradictory. Particularly discrepant are data on pathogens of this genus causing plant soft rot (LELLIOTT and DICKEY, 1984 ; SKERMAN *et. al.*, 1980 ; WALDEE, 1945).

The proposal to place the agents of plant soft rot into separate genus *Pectobacterium* was not generally accepted since it did not resolve the contractions inside genus *Erwinia*. There is need of a more thorough study of these pathogens to determine their phylogenetic affinity with each other and other members of the genus *Erwinia*.

The present work gives comparative characteristics of fatty acid composition of cell lipids of plant soft rot agents, *E. amylovora* and *Escherichia coli* as well as other members of genus *Erwinia*.

The strains used in this study are presented in Table 1.

Table 1. Bacterial strains used in the study

Genus, species, strains numbers, obtained from

Erwinia carotovora subsp. *atroseptica* :
21A, 27A, 30A, 36A, 39A. From Dr. A.N. EVTUCHENKOV, Byeloruss Univ.
NCPPB 549.
NCPPB 3386, NCPPB 3390, NCPPB 3404, NCPPB 3406.

E. carotovora subsp. *betavasculorum* :
NCPPB 2792, NCPPB 2793, NCPPB 2795, ICPPB 4225.

E. subsp. *carotovora* :
ATCC 15713 (Solanum tubesorum), NCPPB 392 (Cucumis sativus), NCPPB 468 (Hyacinthus orientalis), NCPPB 438 (Iris sp.), NCPPB 547 (Percea americana), NCPPB 550 (Nicotiana tabacum).

NCPPB 1744 (Daucus carota v. sativa), EC 153 (Capsicum annuum). From Dr. Yu. K. FOMICHOV, Byeloruss Univer.

67, 216, 258, 246 (Brassica oleraceae). From Dr. I. V. VORONKEVICH. Scientific Research Institute of Phytopathology, Moscow.
48, 53 (Iris sp.). From Dr. L. K. PAVLOVA-IVANOVA, Botanical gardens, Moscow.
71, 92, 133 (Brassica oleraceae).

718, 921 (Daucus carota v. sativa). From Dr. R. HALACHYAN, Institute of Microbiology Academy of Science of Armenia.
EC 1. From Dr. M. GOTO, Japan.

73 (Calla L.), 741 (Hyacinthus orientalis), 144a (Brassica oleraceae). From Dr. L.V. KABACHNAYA, Institute of Microbiology and Virology Academy of Science, Ukraine.

E. chrysanthemi :
I subdivision : B-27 (Dieffenbachia amoena), 73 (D. maculata) ;
II subdivision : NCPPB 516 (Partenium argentatum) ;
III subdivision : C 191 (Euphorbia pulcherima) ;
IV subdivision : 248 (Philodendron selloum) b-73 (Syngonium podophyllum), B-100 (Dracaena marginata), NCPPB 898 (Pelargonium capifatum), 3 (Cyclamen sp.), A-15 (Ipomoea batatas), G 18-3 (Ananas comosus) ;
V subdivision : 277-3 (Lycopersicon esculentum), NCPPB 1955 (Dahlia pinnata). From Dr. R.S. DICKEY, USA, Comell University
VI subdivision : NCPPB 2511, ICPB 2349 = Dye EC 53, ICPB 6353 = Dickey 221, ICPB 6357 = Dickey 414.
NCPPB 402 (Chrysanthemum morifolium).

E. cytolitica 8449, NCPPB 1065, G 147 (Zea mays), *E. carnegieana* : NCPPB 671. From Dr. LAZAR, Inst. of Biology, Romania.

E. amylovora : 595. From Dr. R.A. LELLIOTT, England.

E. cypripedii : NCPPB 3004, *E. herbicola* : NCPPB 2971, *E. nigrifluens* : NCPPB 564, *E. rhapontici* : NCPPB 1578, *E. rubrifaciens* : NCPPB 2021, *E. tracheiphila* : PDDCC 5845, *E. lathyri* : G 155, G 157. From Dr. DE LEY.

Escherichia coli : ATCC 11775.

MATERIALS AND METHODS

Bacteria have been cultivated on potato agar at 28°C. Fresh bacterial cells at stationary growth phase were used in experiments. Methanolysis of bacterial mass, the extraction of methyl esters of fatty acids, their evaporation were carried out according to BRIAN and GARDNER (1967).

Fatty acid methyl esters were analyzed on "Chrom 5" gas chromatograph. The column packed with 5% SE-30 on chromatone N-AW-DMCS (160-200 mesh). Gas chromatographic peaks were primarily characterized by comparing retention time of unknown peaks to those of fatty acid methyl esters standards. The identities of unsaturated and cyclopropane fatty acids were confirmed by procedure of BRIAN and GARDNER (1968). The quantitative content of fatty acids was expressed in percentage from total area of peaks.

RESULTS

The results obtained show that the members of *E. carotovora* and *E. chrysanthemi* cultivated under similar conditions have similar but rather different fatty acid composition of cell lipids. Cell lipids of these pathogens contain fatty acids having from 12 to 18 carbon atoms (Tables 2, 3). The main fatty acids of cell lipids of *E. carotovora* and *E. chrysanthemi* are hexadecanoic, hexadecenoic and octadecenoic acids.

Large amount of unsaturated fatty acids were found in the composition of cell lipids of both species. The representatives of both species do not synthesize cyclopropane fatty acids. We consider that low content of tetradecanoic acid in the composition of cell lipids and comparatively high amount of cell of dodecanoic acid are the most important characteristics which essentially distinguish *E. carotovora* from other members of the genus *Erwinia*. In cell lipids of *E. chrysanthemi* dodecanoic acid was not found or its content was very low.

For the differentiation of *E. carotovora* ssp. *carotovora* and *E. carotovora* ssp. *atroseptica* isolated from potato, DE BOER *et al.* (1986) proposed to use the ratio of some cellular fatty acids. The results we obtained show that majority of strains of *E. carotovora* ssp. *carotovora* can be separated from *E. carotovora* ssp. *atroseptica* by quantitative content of some fatty acids. However, *E. carotovora* ssp. *atroseptica* practically did not differ from *E. carotovora* ssp. *betavasculorum* by the quantitative content of cellular fatty acids.

Table 2

Fatty acid	Erwinia carotovora ssp. carotovora (23)	Erwinia carotovora ssp. atroseptica (10)	Erwinia carotovora ssp. beta-vasculorum (4)	Erwinia amylovora 595	Escherichia coli 17775
12:0	4,8	5,8	5,6	5,2	3,7
13:0	tr.	tr.	tr.	-	tr.
14:0	1,2	3,1	1,9	5,6	8,0
15:0	3,1	4,1	1,9	0,9	1,0
30H-14:0	4,6	5,6	6,6	8,1	6,3
16:1	33,0	33,7	34,2	25,0	14,4
16:0	31,6	31,8	32,8	29,5	27,9
17:1	0,6	0,6	1,1	-	-
17 cyc.	-	-	-	9,3	9,1
17:0	1,2	0,8	1,6	-	-
18:1	18,5	13,3	13,2	18,8	25,1
18:0	1,4	1,2	1,1	1,1	2,7
19 cyc.	-	-	-	-	1,8
S =	53,4	47,6	48,5	39,3	39,5
16:1/18:1	1,8	2,5	2,6	1,3	0,6

E. chrysanthemi strains belonging to subdivisions I - V (DICKEY *et al.*, 1979) have similar fatty acid composition of cell lipids and only pv. *paradisiaca* slightly differ from them. In the representative of this pathovar the dodecanoic acid was discovered in cell lipids in contrast to other members of the species, but its amount was lower than that of tetradecanoic acid and considerably lower than in *E. carotovora.* The fatty acid composition of cellular lipids of *E. cytolitica* is similar to that of *E. carotovora. E. carnegieana* NCPPB 671 differs by cellular fatty acid composition both from *E. carotovora* and *E. chrysanthemi* (Table 3).

Table 3

Fatty acid	Erwinia carotovora 15713	Erwinia chrysanthemi I-V subdi-visions	VI subdi-visions	Erwinia cytolitica 8449	Erwinia negieana 671	Erwinia amylovora 595
12:0	4,6	-	1,3	5,5	5,2	5,2
13:0	tr.	-	-	0,6	-	-
14:0	1,1	5,6	7,6	1,4	6,4	5,6
15:0	3,1	2,3	2,3	3,1	tr.	0,9
30H-14:0	4,6	3,2	3,0	4,5	7,4	8,1
16:1	34,3	35,0	32,9	32,7	21,2	25,0
16:0	32,1	35,1	33,2	31,1	31,8	29,5
17:1	0,6	0,8	1,5	0,8	-	-
17 cyc.	-	-	-	-	11,3	9,3
17:0	1,2	1,0	0,9	2,2	-	-
18:1	18,5	16,9	16,3	16,7	15,6	18,8
18:0	1,4	1,9	1,8	1,4	1,0	1,1
19 cyc.	-	-	-	-	-	4,3
S =	53,4	51,9	49,2	50,2	36,8	39,3
16:1/18:1	1,9	2,0	2,0	2,0	1,4	1,3

The members of *E. carotovora* and *E. chrysanthemi* differ by fatty acid composition of cell lipids both from *E. amylovora* and *Escherichia coli*, type representatives of genus *Erwinia* and family *Enterobacteriaceae* respectively and from other species of genus *Erwinia*.

In contrast to the agents of plant soft rot they synthesize cyclopropanic fatty acids, have high content of tetradecanoic acid as compared with dodecanoic acid ; in spectra of their lipids saturated acids prevail.

Thus the results obtained allow to conclude that *E. carotovora* and *E. chrysanthemi* differ from each other as well as from other members of genus *Erwinia* by fatty acid composition of cell lipids.

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Establishment of a fatty acid data base for automated and rapid identification of strains from the genus *Xanthomonas*

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Abstract

More than 1400 *Xanthomonas* strains representing all seven *Xanthomonas* species and over 130 *Xanthomonas campestris* pathovars were analyzed by gas-liquid chromatography of whole-cell fatty acid methyl esters. At least 65 fatty acids have been found within the genus *Xanthomonas*. These fatty acids fall within the following categories: saturated, unsaturated, methyl-branched, hydroxy, hydroxy-branched, cyclopropane as well as several fatty acids of unknown identity. Nine fatty acids, i. e. 11 : 0 iso, 11 : 0 iso 3OH, 12 : 0 3OH, 13 : 0 iso 3OH, 15 : 0 iso, 16 : 1 cis 9, 16 : 0, 17 : 1 iso F and 17 : 0 iso appeared in more than 99% of the strains tested and are considered as the most common fatty acids for the genus *Xanthomonas*. Among these, the fatty acids 11:0 iso, 11:0 iso 3OH and 13:0 iso 3OH differentiate the genus *Xanthomonas* from other phytopathogenic bacteria. Cluster analysis using the Microbial Identification System (Microbial ID, Inc., Newark, Delaware, USA) revealed 31 major fatty acid clusters among the 966 *Xanthomonas* strains investigated. The established fatty acid database allows rapid identification of unknown strains at genus, species and often at pathovar level. Comparison of fatty acid data with the results of phenotypic analysis, protein electrophoretic pattern analysis and DNA-DNA hybridization revealed that fatty acid profiling is not only useful for rapid identification of unknown isolates but also as a chemotaxonomic marker.

Introduction

The genus *Xanthomonas* contains mainly bacterial plant pathogens. At present, 7 species are established within this genus, i. e. *X. albilineans*, *X. axonopodis*, *X. campestris*, *X. fragariae*, *X. maltophilia*, *X. oryzae* and *X. populi* (Bradbury, 1984; Willems et al., 1987; Swings et al., 1983, 1990; Ridé and Ridé, 1992). Over 140 pathovars have been described within the species *X. campestris*. At present the pathovars of *X. campestris* can not be differentiated on the basis of standard physiological and biological tests. The actual assignment of *X. campestris* isolates to a particular pathovar should be done in terms of phytopathological specilization. This is practically impossible if the normal host plant of the isolate is not known. On the basis of present taxonomy of the genus *Xanthomonas*, non-pathogenic or avirulent xanthomonads can not be identified.

In order to improve the taxonomy of the genus *Xanthomonas* as well as to achieve accurate and rapid identification of xanthomonads, several other fingerprinting techniques such as SDS-PAGE protein profiles, DNA-DNA hybridization and fatty acid analysis have been used to study *Xanthomonas* strains (Stead, 1989; Vauterin et al., 1991, 1992; Yang et al., in press). Fatty acid fingerprinting is a relative simple, rapid and highly reproducible method. At present an automated identification system based on fatty acid composition (Microbial Identification System) is available. Fatty acid composition of well characterized strains can be stored in the computer as an entry of fatty acid database. Fatty acid profiles of an unknown strain can be analyzed and identified with the established fatty acid database. The present paper summarizes the results of fatty acid analysis of over 1400 *Xanthomonas* strains. Part of the results has been published previously (Yang et al., in press).

Materials and Methods

Strains. More than 1400 *Xanthomonas* strains which covered all the currently recognized 7 *Xanthomonas* species, 135 *X. campestris* pathovars and two pathovars of *X. oryzae* as well as some non-pathogenic xanthomonads were studied.

Gas-liquid chromatographic analysis of the fatty acid methyl esters. The procedures for the culture of bacteria, extraction of fatty acid methyl esters, gas-liquid chromatographic determination of fatty acid methyl esters, numerical analysis of the fatty acid profiles and the generation of fatty acid database using the Microbial Identification System have been described elsewhere (Vauterin et al., 1991, 1992; Yang et al., in press).

Results and Discussion

Fatty acids found. *Xanthomonas* strains contained at least 65 different fatty acids. These included saturated, mono-unsaturated, methyl-branched, hydroxy, branched-chain hydroxy, cyclopropane fatty acids as well as several fatty acids with unknown identity.

Most *Xanthomonas* strains shared a common qualitative pattern. Nine fatty acids appeared in at least 99% of the strains tested (11 : 0 iso, 11 : 0 iso 3OH, 12 : 0 3OH, 13 : 0 iso 3OH, 15 : 0 iso, 16 : 1 cis 9, 16 : 0, 17 : 1 iso F and 17 : 0 iso) and were considered as the most common fatty acids for the genus *Xanthomonas*. Among them 11:0 iso, 11:0 iso 3OH and 13:0 iso 3OH are characteristic for the genus *Xanthomonas* and serve as useful criterion to differentiate *Xanthomonas* from other bacteria. The most abundant fatty acids were 15:0 iso and 16:1 cis 9.

Relationship among *Xanthomonas* strains based on the FAME composition. Cluster analysis revealed 31 Major FAME clusters among 966

Xanthomonas strains investigated. The species *X. albilineans*, *X. axonopodis*. *X. fragariae*, *X. maltophilia* and *X. populi* each constitutes a separate cluster, whereas two clusters were formed within the species *X. oryzae*, corresponding to *X. o.* pv. oryzae and *X. o.* pv. *oryzicola*, respectively. The actual species *X. campestris* was heterogeneous and comprised 24 clusters.

In some cases, *X. campestris* pathovars isolated from the related host plants grouped together: four such major groups were found. The first group included the following *X. campestris* pathovars from grasses: *X. c.* pvs. *graminis, poae, phleipratensis*. The second group contained *X. campestris* pathovars from cereals: *X. c.* pvs. *cerealis, hordei, undulosa, secalis* and *translucens*. The third group comprised all the six *X. campestris* pathovars from crucifers: *X. c.* pv. *aberrans, armoraciae, babareae, campestris, incanae* and *raphani*. Many *X. campestris* pathovars isolated from legumes were grouped together: *X. c.* pvs. *rhynchosiae*, *phaseoli* var. *fuscans*, *vignicola* FAME group A, *sesbaniae, glycines, alfalfa, alangii and phaseoli* FAME group A.

Homogeneity of *Xanthomonas* species and *Xanthomonas campestris* pathovars. In general, *Xanthomonas* species and most *X. campestris* pathovars were homogeneous although one or a few atypical strains were frequently encountered. Some *Xanthomonas campestris* pathovars are heterogeneous For example, *X. c.* pvs. *vasculorum*, *citri*, *phaseoli* and *vignicola* each formed two or more FAME subgroups.

Generation of fatty acid database. The fatty acid database generated allows rapid identification of unknown isolates at genus, species and often pathovars.

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Analysis and characterization of plasmids in *Xanthomonas campestris* pv. *oryzae*

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ABSTRACT

A collection of *X. c.* pv. *oryzae* strains differing in geographical origin was screened for the presence of plasmids. Out of seventeen isolates of *X. c.* pv. *oryzae*, fourteen harbored plasmids of which two isolates (XOP5, XOC26) had two plasmids each and one isolate (XOR20) harboured three plasmids. The remaining isolates contained a single plasmid of identical mobility. The restriction fragment pattem of the plasmid was distinct in each isolate. They were classified under three groups based on cluster analysis using unweighted pair group method with averages (UPGMA). From the different plasmids, the plasmid pMA36 (*X. c.* pv. *oryzae* XOC36) was further studied. This plasmid was cured by acridine orange at the frequency of 10%. The cured strain was transformed with pMA36 at a frequency of 2.3×10^2 transformants/µg of plasmid DNA. Plasmid cured strain was virulent on rice but symptom development was remarkably delayed when compared to wild and transformed strains. The plasmid influenced pathogenicity of *X. c.* pv. *oryzae*.

KEYWORDS

Plasmid, curing, transformation, pathogenicity, *Xanthomonas campestris* pv. *oryzae*.

INTRODUCTION

Xanthomonas campestris pv. *oryzae* (*Xco*) is the causal agent of bacterial blight of rice, a perennial disease of major significance in rice growing countries (RANGASWAMI, 1988). Though several factors have been attributed to pathogenicity including extracellular polysaccharides (ANGADI, 1978), the mechanism of pathogenicity of *Xco* on rice still remains an enigma. The DNA of *Xco* is reported to contain genes isofunctional with *X. c.* pv. *campestris* genes required for pathogenicity (TODD *et al.*, 1990).

A 2.5 Kb EcoR1 fragment from the chromosome of *X.c.* pv. *oryzae* has been reported to code for avirulence (*avr*10) corresponding to *Xa*-10 resistant gene (KELEMU & LEACH, 1990). Plasmids are known to code for a variety of functions including pathogenicity in plant pathogenic bacteria (ULAGANATHAN & MAHADEVAN, 1985). Isolates of *Xanthomonas campestris* pathovars collected from different geographical regions were screened for plasmids. One of the plasmids from *X.c.* pv. *oryzae* was studied in depth.

MATERIALS AND METHODS

Strain

Seventeen isolates of *Xanthomonas campestris* pv. *oryzae* isolated from infected rice leaves and confirmed on the basis of pathogenicity test, morphology and biochemical tests (BRADBURY, 1986).

Plasmid Isolation

After several preliminary trials, the modified EMBO method was routinely used (MAHADEVAN & ULAGANATHAN, 1992).

Restriction Enzyme Digestion of Plasmid DNA

Restriction enzymes and λ DNA were obtained from Promega, Leiden, the Netherlands and used according to the manufacturer's recommendation.

Analysis of Fragments and Cluster Analysis

The molecular weights of the fragments were calculated by comparison of relative mobility with λ Hind III fragments. Cluster analysis was carried out according to GOWER (1985).

Plasmid Curing

In order to obtain plasmid free derivatives, curing experiment was carried out by growing the cells in Luria broth supplemented with acridine orange (10-50 μ g/ml).

Plasmid transformation

The transformation of plasmid pMA36 into the cured strain was performed with some modification of the method described by ATKINS *et al.* (1987).

Pathogenicity test

The pathogenicity test for *X. campestris* pv. *oryzae* (wild, cured, cured strains transformed with plasmid pMA36 and acridine orange treated plasmid harbouring strain) was performed on the rice cultivar ADT 36. Two inoculation methods were followed : (1) to clipping method (2) pin prick method (DEVADATH, 1985). Appropriate controls were maintained. Observations were made from the 10th day by measuring lesion length of 25 inoculated leaves in each set and the results were interpreted based on standard evaluation system for rice (ANONYMOUS, 1988).

RESULTS AND DISCUSSION

The modified EMBO procedure facilitated the detection of both large and small plasmids in all the strains. *X.c.* pv. *oryzae* displayed little diversity in the distribution of plasmids. Of the 14 plasmids bearing isolates, XOR20 and XOC26 had more than one plasmid and other isolates harboured a single plasmid. ULAGANATHAN & MAHADEVAN (1991) reported 1 to 2 plasmids per isolate. The remaining isolates contained a single plasmid of identical mobility. Ten isolates of *X.c.* pv. *oryzae* containing a single plasmid were selected and their plasmids were digested with EcoRI. All the 10 isolates of *X.c.* pv. *oryzae* had distinct plasmid restriction fragment pattern. There were distinct variations in the intensity of fluorescence among different bands of the same isolate and among different isolates. The plasmids endrogram derived from the similarity coefficients grouped *X.c.* pv. *oryzae* plasmids into three distinct groups. The first group contained XOA9 and XOK3. This group showed 36% similarity with group II and 80% similarity with group III. The second group consisted of XOC43 and XOC4. The remaining 6 isolates constituted a third group. The most closely related isolates among the 10 isolates were XOM7 and XOL1, showing more than 98% similarity. XU & GONZALEZ (1991) have shown that among the 26 strains of *X.c.* pv. *oryzae*, 20 strains harboured indigenous plasmids and could be divided into three distinct groups.

Four groups were identified based on RFLP analysis and bacteriocin typing. Five sub groups were found based on plasmid content, RFLP analysis and bacteriocin typing.

Attempts have already been made to cluster different isolates of *X.c.* pv. *oryzae* based on physiological, biochemical characters and sensitivity to bacteriophages. TSUCHIYA *et al.* (1982) compared the physiological and morphological characteristics of wild virulent strains and induced mutants.

At the International Rice Research Institute, attempts have been made to classify strains from different geographical regions by their reaction to phages as an aid to epidemiological studies of bacterial blight (VERA CRUZ & MEW, 1989). These attempts have the inconvenience of being based on phenotypic characteristics rather than genetic relatedness. The grouping of strains by finger print analyzis is based on genetic diversity which could be more specific than the phenotypic characteristics, sinced phenotype is trongly influenced by environment. This could be one of the reasons why our grouping did not correlate with geographical origin of the isolates.

A single plasmid was selected from XOC36 for further characterization. Plasmid curing did not affect growth, colony morphology, polysaccharide and pigment production. Plasmid pMA36 was transformed into cured strain at a frequency of 2.3 x 10^2. The lower transformation frequency may be due to the large size of the plasmid. Transformation of indigenous plasmid into *X.c.* pv. *malvacearum* has been reported (GABRIEL, 1984). The plasmid cured strain was sensitive to ampicillin. When the plasmid was transformed into the cured strain, the ampicillin resistance was regained indicating that plasmid pMA36 codes for ampicillin resistance.

Symptoms were discernible after 6-7 days in plants inoculated with wild and transformants. The onset of symptoms was delayed by 6-8 days in the plants inoculated with cured strain i.e., symptoms appeared only 13-14 days after the inoculation. Disease intensity was 50% (scale,5) on 16th day for wild and transformants whereas it was only 15% to 18% (scale,3) in the case of cured strain. To cause complete wilting of the leaf, wild and transformants needed 22 days whereas the cured strain caused 50% disease intensity only on the 22th day.

There was no significant difference in disease intensity among the wild and transformants and acridine orange treated plasmid harbouring strain. The delayed symptom development by the plasmid cured strain indicates that pMA36 influences pathogenicity. The acridine orange induced mutation on chromosome is ruled out since the acridine orange treated plasmid harbouring strain was a pathogenic as the wild strain. The effect may be explained by the properties of plasmid pMA36 that support cell metabolism with additional functions required for contact with the plant host cell.

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Cluster analysis of *Xanthomonas campestris* pv. *vesicatoria* strains based on carbon source utilization patterns

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ABSTRACT

Strains of *Xanthomonas campestris* pv. *vesicatoria* (Xcv), the etiological agent of bacterial spot of tomato and pepper, were tested for their ability to utilize the 95 different carbon sources available in the GN MicroPlate™ (Biolog Inc., Hayward, CA). The dendogram generated from cluster analysis of utilization patterns indicates that Xcv is a diverse group of strains. Xcv formed several clusters that spanned seven of 11 pathovars tested. Cluster analysis segregated most starch hydrolytic Xcv strains from starch-negative Xcv strains. The simplicity and rapidity of testing the utilization of substrates by a large group of strains make the Biolog system useful for characterizing Xcv strains. Also, because this system provides information on the similarity of strains, it might prove useful for bacterial classification.

KEYWORDS

Xanthomonas campestris pv. *vesicatoria, cluster analysis, carbon substrate utilization, systematics.*

INTRODUCTION

Xanthomonas campestris pv. *vesicatoria* (Xcv) is the causal agent of bacterial spot, a serious disease of tomato (*Lycopersicon esculentum* Mill.) and pepper (*Capsicum annuum* L.) in tropical and sub-tropical regions of the world (GOODE & SASSER, 1980). The Xcv population is apparently composed of phenotypically different strains (COOK & STALL, 1982 ; MINSAVAGE *et al.*, 1990). Strains from Argentina and Brazil were virulent on the tomato genotype 'Hawaii 7998' (WANG *et al.*, 1990) did not countain the avirulence gene avrRxv (WHALEN *et al.*, 1988) degraded pectin (BEAULIEU *et al.*, 1991) and hydrolyzed starch (STALL *et al.*,

unpublished). To determine the phenotypic composition of Xcv populations in different regions where tomato and pepper are cultivated, we have characterized Xcv strains by SDS-PAGE of cellular proteins and by their ability to hydrolyse starch or degrade pectin. To rapidly analyze a large number of metabolic traits, we used the Biolog system (Bochner, 1989) which allows detection of substrate utilization of 95 carbon sources present in the GN MicroPlate™ by the reduction of a tetrazolium dye. Upon oxidation of the carbon-source by the bacterium, a purple color is produced which can be read with a microplate reader. The resulting metabolic fingerprint can be stored and analyzed by a computer.

Materials and Methods

Two-hundred and five Xcv strains originating from countries of the Americas, Asia, Europe and the Pacific, as well as strains from 11 pathovars of *X. campestris*, *Pseudomonas syringae* pv. *syringae*, *P. solanacearum*, and *Erwinia herbicola* were used in the present study. Bacteria stored at -80 C were initially grown on nutrient agar (Difco Laboratories, Detroit, MI 48232) before being transfered to trypticase soy agar (BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD 21030). The TSA plates were incubated for 18 h at 28C, and the bacteria were harvested and suspended in sterile saline (0.85% NaCl) to an O.D. of A_{590nm} = 0.17 - 0.19. The GN MicroPlate™ (Biolog Inc., Hayward, CA 94545), which contains 95 substrates and includes amino acids, carboxylic acids, and carbohydrates, was inoculated with the bacterial suspension (0.15 ml/well) and incubated for 24 h at 28C. The resulting utilization patterns were read at A_{590} with an automated plate-reader (EAR 400 AT, SLT-Labinstruments, A-5082 Grödig/Salzburg, Austria) and downloaded to a computer for compilation of a database. The database was subjected to cluster analysis using the MLCLUST program of the MicroLog™ (Biolog) to determine strain relationships.

Results and Discussion

With the exception of two Xcv strains which did not oxidize any of the substrates after the 24 h incubation recomended by the manufacturer, cluster analysis of utilization patterns indicates that Xcv strains forms several clusters which include seven of the 11 pathovars tested.

According to SDS-PAGE of cellular proteins, our collection of Xcv strains is composed of two major groups. The larger group (i.e., A) is made up of 148 strains which share a unique protein (32 kDa) and are unable to utilize starch. The second group of strains (i.e., B) has a smaller protein (27 kDa) and 90 % of these strains hydrolyze starch. With a few exceptions, cluster analysis resulted in the segregation of group B strains from group A.

Analysis of strains isolated from different fields within the same area, formed a tight cluster. This was the case for group A strains from Guadeloupe and group B strains from Costa Rica (data not shown). Apparently, the pathogen in these fields exhibit limited polymorphism, suggesting that the structure of the population is clonal in nature. The simplicity and rapidity of testing the utilization by a large group of strains of a panel of substrates makes the Biolog system useful for characterization of Xcv strains. In addition, because this system provides information on the similarity of strains, it should prove useful for bacterial classification studies.

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Identification and characterization of plant pathogenic pseudomonads with biolog Microplates TM and Microlog TM

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1 Abstract

The Biolog Microplate test panels and Micrilog software have been designed for quick identification of a broad range of bacterial species according to their ability to metabolize 95 carbon sources. We tested whether this method is useful for a quick characterization of three incompletely described pathogens:

The causal agent of bacterial blood disease of banana (BDB) can neither be differentiated from *Pseudomonas solanacearum* (*Pso*) by symptoms on the host nor by tests with polyclonal antibodies. Our results showed that these two pathogens could be distinguished by their metabolic capabilities.

The incitant of a blight disease on *Coriandrum sativum*, a non-fluorescent pathogen belonging to the *Pseudomonas syringae* group, was recently discovered in Germany. The isolates of this pathogen could clearly be distinguished from other *P. syringae* strains by a uniform metabolic pattern.

The incitant of basal glume rot of cereals, *Pseudomonas syringae* pv. *atrofaciens* (*Psa*), can be distinguished from *P. s.* pv. *syringae* (*Pss*) only by its capability to infect the ears of cereals. We tested whether these pathogens could also be differentiated by physiological means. In our studies, isolates of *Pss* from different hosts did not possess similar metabolic capabilities, whereas all isolates of *Pss* and *Psa* from cereals did.

Based on results from three different plant pathogenic bacteria the Biolog system proved to be a reliable, specific, moderately priced and time-saving method.

TM: Trademark of Biolog Inc., Hayward, California, USA.

2 Introduction

The Biolog system has been developed during the last ten years for the identification of bacteria according to their metabolic "breathprints" (BOCHNER, 1989). The system consists of the Microplate test panels with 95 different carbon sources and the Microlog computer software which recognizes bacteria by their typical pattern of metabolism.
We evaluated this system for the identification and characterization of different plant pathogenic pseudomonads.

3 Materials and Methods

Selected strains of *P. solanacearum* (*Pso*), blood disease bacteria (BDB), *Pseudomonas syringae* pv. *atrofaciens* (*Psa*), *Pseudomonas syringae* pv. *syringae* (*Pss*), and *Pseudomonas syringae* strains from coriander were grown for 24 h on Tryptic Soy Agar (TSA). The cells were removed from the plates with a sterile swob and suspended in sterile saline (0.85% NaCl). The inoculum concentration was adjusted to an optical density of 0.15 at 590 nm, and 150 µl were filled into the cavities of the Microplates. After incubation for 24 h at 28°C, wells showing a positive reaction by change of colour were recorded. The pattern of reaction was analyzed by the Microlog software (release 2.0).

4 Results

4.1 *Pseudomonas solanacearum* (*Pso*) and blood disease bacteria (BDB)

The Biolog system identified eight from nine isolates of *Pso* with the classification "good" and one with the classification "poor". The latter had 49% similarity with the software standard of *Pso* (Table 1). Only two out of nine isolates of BDB were identified by Biolog as *Pso* with only 27% similarity in each case. The other isolates of BDB were classified as various species of *Pseudomonas*.
The *Pso* strains could metabolize the carbon sources maltose (well B10), acetic acid (D 1), cis-aconitic acid (D 2), citric acid (D 3), D-galacturonic acid (D 6), D-gluconic acid (D 7), D-glucuronic acid (D 9), propionic acid (E 8), D-saccharic acid (E 10) and gamma-amino butyric acid (G12), whereas the BDB isolates showed no or only weak growth on these substances.

Table 1: Metabolic pattern of 9 isolates each of *Pso* and BDB in Microplates (95 different carbon sources). The number indicated in each well represents the number of positive reactions (BDB upper row, *Pso* lower row following each letter).

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	0 0	0 0	0 0	0 0	9 9	6 9	0 0	0 3	0 0	0 0	0 0	0 0
B	0 0	6 8	0 0	2 5	0 0	7 9	0 2	0 0	0 0	2 9	0 4	0 0
C	0 0	0 0	5 7	0 0	0 0	0 4	1 5	4 8	0 0	0 0	8 9	8 6
D	1 9	0 9	1 8	0 0	0 2	0 8	0 9	0 1	0 9	6 4	7 9	3 1
E	0 0	0 0	5 8	8 9	0 0	9 9	0 0	1 9	8 9	1 9	5 1	9 9
F	9 9	9 9	1 5	6 9	9 9	9 9	6 9	9 9	9 9	9 9	0 0	1 1
G	5 9	0 0	0 5	0 2	0 2	9 9	9 6	5 6	9 9	4 9	0 0	0 6
H	4 4	2 6	0 0	0 0	0 0	0 0	0 0	0 0	5 8	0 3	0 1	0 1

Table 2: Metabolic pattern of 11 *P. syringae* strains isolated from coriander in Microplates (95 different carbon sources). The number indicated in each well represents the number of positive reactions.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	0	0	0	0	11	11	0	0	0	11	10	0
B	0	11	0	11	0	11	11	0	0	6	11	11
C	0	0	11	10	0	11	11	0	0	0	11	4
D	8	11	11	9	11	10	11	11	11	0	1	0
E	0	0	0	11	0	0	10	7	4	11	0	11
F	11	11	10	5	11	11	11	11	11	11	0	11
G	3	0	1	0	0	11	0	6	10	6	0	11
H	0	11	11	0	0	0	0	0	10	11	3	11

4.2 *Pseudomonas syringae* isolated from coriander

According to Biolog, all tested isolates from coriander reacted like *P. syringae* pv. *pisi* (*Psp*). Nevertheless, the tested strains consistently metabolized carbon sources on which *Psp* did not grow, i.e. Tween-80 and glucose-6-phosphate (Table 2).

4.3 *Pseudomonas syringae* isolated from cereals and other hosts

The strains were identified by Biolog as follows:

Table 3: Strains of *Psa* and *Pss* from different hosts, their probable relatedness and pathovar identification according to the Biolog system

Strain	Host	Origin	Pathovar identification by Biolog	Distance to identified pathovar
Pseudomonas	*syringae* pv.	*atrofaciens:*		
NCPPB 2612	wheat	New Zealand	aptata	1.7
GSPB 1723	wheat	Germany	aptata	0.9
GSPB 1742	barley	Germany	aptata	0.9
GSPB 1873	wheat	Bulgaria	aptata	2.6
GSPB 1875	wheat	Bulgaria	aptata	1.5
GSPB 2067	wheat	South Africa	aptata	1.9
GSPB 2074	wheat	South Africa	aptata	1.3
GSPB 1576	barley	USA	aptata	1.0
Pseudomonas	*syringae* pv.	*syringae:*		
NCPPB 2842	wheat	USA	aptata	0.8
GSPB 1569	barley	USA	aptata	3.0
GSPB 1570	barley	USA	aptata	4.3
GSPB 839	cherry	Germany	delphinii	8.5
GSPB 860	cherry	USA	lachrymans	0.9
GSPB 1004	lilac	Germany	aptata	2.4
NCPPB 281	lilac	U:K:	pisi	9.6
NCPPB 1053	millet	Ethiopia	syringae	1.1
NCPPB 2260	*Prunus*	Yugoslavia	atrofaciens	3.6
GSPB 1150	bean	Germany	aptata	1.4

All isolates of *Psa* and of *Pss* which originated from wheat and barley were most similar to *P. s.* pv. *aptata*. This pathovar is closely related to *Psa* and causes symptoms on wheat after artificial inoculation. Isolates of *Pss* from other hosts were identified as various pathovars of *P. syringae*.

5 Discussion

P. solanacearum and blood disease bacteria

The incitant of blood disease is closely related to *Pso*. The two organisms can neither be distinguished by symptom expression on the host plant nor by traditional tests using polyclonal antibodies. With the Biolog method we found several carbon sources which differentiate the two pathogens. These results confirm the data obtained with "monospecific antibodies" (BAHARUDDIN et al., 1992). Further tests are necessary to determine whether BDB should be classified as a new biovar of *Pso* or whether even the original name *Pseudomonas celebensis* should be re-installed.

P. syringae isolated from coriander

The incitant of a blight disease on coriander, a non-fluorescent pathogen belonging to the *P. syringae* group, was recently discovered in Germany. The eleven strains tested had a similar metabolic pattern. It differed from *Pseudomonas syringae* pv. *pisi* and was dissimilar to any other pathovar. These findings support other results from TOBEN et al. (1992) who suggested to classify these strains as a new pathovar of *P. syringae*.

P. syringae isolated from cereals

During the last 20 years strains of *P. syringae* from cereals have been reported in many countries. Several workers classified their isolates as *Pss* and not as *Psa* following OTTA's argument (1977) who found no differences between his isolates and *Pss*. In compendia (e.g. WIESE, 1987) the two pathovars of *P. syringae* are distinguished only by the different symptoms they produce on the host. Our studies showed, that although *Pss*-strains comprise a group of bacteria with a very heterogenous metabolic pattern, all isolates of *P. syringae* from cereals possess similar metabolic capabilities. The fact that none of the tested strains was identified as *Psa* is probably due to the incompleteness of the software. However, it might be impossible to distinguish closely related pathovars of *P. syringae* with this system

as their physiological capabilities are not necessarily correlated with host specificity.

Compared to other methods for the identification and characterization of bacteria, e.g. fatty acid analysis (SASSER, 1990), the Biolog system gave rather satisfactory results, especially in view of its moderate price and the quick and easy procedure. However, as stated by JONES et al. (1991), this system is not suited for the differentiation of some closely related pathovars of *P. syringae.*

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Development of serological tools for the detection of *Xanthomonas* species

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ABSTRACT

Polyclonal antisera were produced against *Xanthomonas campestris* pv *begoniae* (XCB), *Xanthomonas campestris* pv *dieffenbachiae* (XCD), and *Xanthomonas campestris* pv *pelargonii* (XCP). These antisera were used to develop double antibody sandwich enzyme linked immuno-sorbent assays (DAS-ELISA) and dot immuno-binding assays (DIBA) for the detection of the pathogens in begonias, anthuriums and pelargoniums. These tests are now available as commercial kits for routine testing laboratories.

KEYWORDS

Xanthomonas, Serology, ELISA, DIBA.

INTRODUCTION

Bacterial wilts caused by *Xanthomonas campestris* pathovars are diseases of major importance, especially in ornamental crops such as pelargonium, begonia and anthurium. Moreover, the industrialization of these crops has led to an increase of the damages induced by these bacteria.

Since no chemical or biological treatments have yet been shown to control these disease, indexing and sanitary selection are the only ways to control them. Thus, the goal of the studies was to develop sensitive and reliable serological assays to detect XCB, XCD and XCP in their respective cultivated hosts.

MATERIALS AND METHODS

* Polyclonal antisera production :

The bacteria were cultivated on YPGA medium (LELLIOTT and STEAD, 1987) during 48 to 72 hrs at room temperature. The isolates used for the immunizations were : for XCB isolates V/10144 and V/10149, for XCD isolate V/11013 and for XCP isolate V/10382.

Cells were harvested at 3500 g for 10 min, and washed three times in sterile distilled water. The final suspension was adjusted photometrically at 10E9 cfu and the cells were heat-killed (2 hrs at 100 °c).

New Zealand white rabbits were hyper-immunized according to a procedure comprising 10-12 intra-veinous injections of 0.5 to 1.5 ml of a 10E9 cfu bacterial suspension (BAYLE, 1988).

The titers of the antisera were determined in immuno-fluorescence assays (IFA) and in indirect antigen-coated plate enzyme linked immuno-sorbent assays (iACP-ELISA).

* Preparation of immuno-reagents :

IgGs were purified by affinity chromatography on a Protein-A Sepharose column (PHILIPPS *et al.*, 1984).

Anti-Xanthomonas purified IgGs were conjugated with alkaline phosphatase (Boehringer, 567752) using glutaraldehyde (AVRAMEAS, 1969).

The goat anti-rabbit antiserum was obtained from BIOSYS (ref. BI 2007) and an alkaline phosphatase conjugate was prepared (GAR-PAL).

* ELISA procedures :

The DAS-ELISA procedure was performed according to CLARK & ADAMS (1977) on Nunc Maxisorp 96 wells microplates. The coating antibodies (150 µl per well) were incubated at 0.5 to 1 µg/ml 2 hrs at 37°C in 50 mM sodium carbonate pH 9.6. The antigens (150 µl per well) were then incubated overnight at +4°C. 150 µl conjugated IgGs per well, prepared in phosphate buffer saline (PBS : 150 mM NaCl, 1.5 mM KH_2PO_4, 10 mM Na_2HPO_4, 2 mM KCl) + 0.05% Tween 20 + 0.2% bovine serum albumine (PBST-BSA) were incubated 2 hrs at 37°C, and the substrate p-nitrophenylphosphate (Boehringer, 107905) was then added at 1 mg/ml in 10% diethanolamine buffer pH 9.8. Three vigorous rinsings were performed between every incubation step.

Readings of the absorbance values were done using a Titertek Multiskan MCC 340 MKII photometer.

In iACP-ELISA tests, bacterial suspensions were coated at 10E6 cfu in sodium carbonate buffer 2 hrs at 37°C. After rinsing, serial dilutions of the antisera to be tested, prepared in PBST-BSA were incubated 2 hrs at 37°C, before deposit of the GAR-PAL conjugate, in the same buffer and following the same incubation procedure.

* <u>DIBA procedure</u> :

1-2 µl of bacterial suspensions and in certain cases plant extracts, prepared in TBS pH 9.5 (20 mM Tris base, 0.5 M NaCl), were directly spotted onto nitrocellulose sheets (Schleicher & Schuell. 405391). The sheets were then incubated in TBS pH 7.5, 0.5% Tween 20, 5% non fat dried milk 30 min at room temperature in order to block the remaining binding sites, before being incubated in the specific-antibody solution prepared in TBS + 0.5% Tween 20 + 1% non fat dried milk, 30 min at 37°C. The antibodies were usually used at 1 µg/ml.

After 3 rinsings in TBS + 0.5% Tween 20 (TBST), the membranes were incubated in 1/1000 (v/v) GAR-PAL diluted in antibody buffer, 30 min at 37°C.

The revelation step was performed using nitro blue tetrazolium and 5 bromo 4 chlroroindolyl phosphate (BLAKE *et al.*, 1984) prepared in 0.1 M Tris, 0.1 M NaCl, 5 mM MgCl2 pH 9.6.

RESULTS

* <u>Titers and specificity</u> :

The antisera titers, determined in indirect IFA, varied between 1/8000 to 1/128000. Each antiserum was tested against different pathovars of *Xanthomonas campestris*, but no obvious cross-reactions could be shown. Nevertheless the antisera to XCB and XCD showed moderate cross-reactivity with *X.C.* pv *oryzicola*, and the three antisera weakly reacted with high concentrations of *X.C.* pv *campestris*, *manihotis* or *incanae*.

No cross-reactions were ever observed with non related phytopathogenic bacteria of the genia *Pseudomonas* and *Erwinia*, non phytopathogenic bacteria of the saprophytic flora, or healthy plant extracts.

* <u>Sensitivity of the DAS-ELISA tests</u> :

The DAS-ELISA sensitivity was determined using either bacterial preparations in PBS buffer, or dilutions of the antigens in crude healthy plant extracts (Table 1).

Table 1 : Sensitivity of the DAS-ELISA tests

Pathogen	XCB	XCD	XCP
Sensitivity (cfu/ml)	10E4	2.5.10E4	5.10E3

* Comparison of the DAS-ELISA test with other routine-testing techniques :

For routine-testing, isolation on non-specific media and IFA were the techniques used until these late years. Thus, our DAS-ELISA tests were compared to these two techniques.

For XCP, about 1000 pelargonium samples were tested, and the results showed a strong correlation between the three techniques however isolation seemed to be the least sensitive.

For XCB and XCD, the tests were performed mainly on artificially infected plants, confirming the results obtained with XCP : perfect correlation between IFA and DAS-ELISA and poor sensitivity of isolation technique (Table 2).

Table 2 : Correlation between isolation, IFA and DAS-ELISA for the detection of *Xanthomonas campestris* pv. *begoniae* (number of samples found positive / number of samples tested) (PHILIPPOT, 1990)

Nature of the sample	Isolation	IFA	ELISA
Healthy plant petiole	0	0	0
Infected plant collar	2/3	2/3	3/3
Infected plant stem	1/6	6/6	6/6
Infected plant petiole	3/11	10/11	10/11

* Rapid testing using nitrocellulose membranes :

The DIBA technique, characterized by short incubation times, and small amounts of reagents needed, gave results similar to those obtained with DAS-ELISA.

DIBA was tested mainly on the XCP model (JACQUET, 1990) and at a lesser extend on XCB. In both cases, the tests sensitivities were the same in DIBA and in DAS-ELISA and no specific difficulties were encountered when running the test, except occasional background problems making uneasy tests, interpretations and the weakness of nitrocellulose sheets requires careful handling.

CONCLUSION

These studies have shown reliability of serological techniques for the detection of phytopathogenic Xanthomonads. In the case of XCP, for instance, the DAS-ELISA technique is nowadays routinely used by most European producers of certified material, and DIBA begins to develop in pelargonium multiplication firms.

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Rapid diagnosis of bacterial wilt

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Abstract

Bacterial Wilt caused by *Pseudomonas solanacearum* is one of the most economically important plant diseases of the tropics, subtropics and warm temperate regions of the world. Previously, identification of this pathogen relied on tedious biochemical analysis taking up to twenty-one days to obtain a result. Several new tests for diagnosing the bacteria, which are far easier and quicker to perform, are currently being produced. We have developed such a test in the form of an Enzyme Linked Immunosorbent Assay (ELISA) which may be used to detect the bacteria in both plant and soil samples in less than a day. A simple indirect ELISA which uses direct binding of whole bacterial cells to a microtitre plate was developed initially for use in the screening of polyclonal and monoclonal antibodies raised against *P. solanacearum*. During its use, parameters (e.g. washing and blocking protocols, plate type) were constantly changed until optimum sensitivity of detection was obtained. The assay was then tested for its possible use as a diagnostic assay for Bacterial Wilt (*P.solanacearum*). When tested with extracts from artificially inoculated plants and also naturally infected potato tubers, the assay was found to be satisfactory: detecting the bacteria even when wilting was not apparent. Also, with only slight alterations to the assay, the bacteria could be detected down to levels as low as 1×10^4 cfu per ml in artificially inoculated soils.

Introduction

Bacterial Wilt caused by *Pseudomonas solanacearum* is a disease widely distributed in tropical, subtropical and warm temperate regions of the world. It is one of the most destructive bacterial diseases of plants, affecting a wide range of economically important crops, including: potato, tomato, tobacco, banana, peanut and ginger. The pathogen also affects many weeds, serving as an inoculum from which the disease may spread. Biochemical identification of *P. solanacearum* is tedious (Hayward, 1964), taking up to twenty-one days to obtain a result. An indirect Enzyme-linked

immunosorbent assay (ELISA) has been developed to replace this and is discussed in this paper. The ELISA was chosen because it is rapid (allowing a large number of samples to be tested in a relatively short period of time), reliable, inexpensive, and easy to perform. Both monoclonal and polyclonal antibodies have been produced to *P. solanacearum* at Rothamsted (Eden-Green & Robinson, unpublished) and have been used successfully in the development of the assay.

Materials and Methods

Production of Antibodies

a) Polyclonal. Polyclonal antibodies were raised in Dutch-Lop Cross rabbits. Initial immunisations of 5 x 10^8 glutaraldehyde fixed whole bacterial cells in Freunds complete adjuvant (Difco Labs) were followed with an identical boost in incomplete adjuvant four weeks later. Rabbits were bled at two weekly intervals via the ear vein, and sera collected.

b) Monoclonal. Monoclonal antibodies were produced by immunising Balb/c mice with multiple injections of 1 x 10^8 glutaraldehyde fixed whole bacterial cells in sterile saline; followed by fusion of their spleen cells with either the myeloma line NSO or P3X63Ag8.653.

Enzyme Linked Immunosorbent Assay

Incubations throughout were at either 37° C for one hour or 4° C overnight. After each stage, excess reagent was removed from the wells by "flicking out". The plates were then washed in three changes of PBS-Tween (phosphate buffered saline plus 0.05% v/v Tween 20), for three minutes each. After washing, the plates were "bang dried" (gently) on tissue paper.

The routine protocol was as follows: Ninety-six well microtitre plates were coated with a suspension of whole bacterial cells (100μl per well) diluted to 1 x 10^8 cfu/ml in 0.05M carbonate coating buffer, pH 9.6. After incubation and washing, 100μl of either a monoclonal or polyclonal antibody diluted in blocking buffer (0.05% v/v Tween 20, 2% w/v polyvinyl pyrrolidone (PVP, Mol.Wt. 44,000) and 0.5% w/v NIDO milk powder (Nestle) in phosphate buffered saline) was added and incubated. After washing, a second antibody, horseradish peroxidase conjugated rabbit anti-mouse or goat anti-rabbit (Sigma) at a 1:2000 dilution in blocking buffer, was added and incubated. Finally, 100μl of TMB substrate (1mg/ml 3,3',5,5'-tetramethyl-benzidine (Sigma), 0.1% v/v hydrogen

peroxide and 10% v/v sodium acetate, pH 5.8 in distilled water) was added (per well) and incubated at room temperature until sufficient colour developed The reaction was stopped by adding 3M sulphuric acid (25μl/well). Results were assessed visually, and then quantified by reading the absorbance at 450nm (including a turbidity correction at 650nm), using a microtitre plate reader (Flow Labs Ltd.).

Optimisation of the ELISA

a) Plate type. Different brands of microtitre plate differ in binding capacity and also in consistency of binding within and between plates. Several commercially available microtitre plates were therefore tested for their binding abilities using a half-log dilution series of whole bacterial cells (1×10^8 - 1×10^1 cfu/ml) to determine the lowest level of bacteria detected by a polyclonal antibody (R283 diluted to 1:5000).

b) Coating buffer. Phosphate buffered saline (PBS), 0.1M citrate buffer, pH 5.0 and pH 3.0, and 0.05M carbonate buffer, pH 9.6, were all compared for their ability to bind bacteria to the microtitre plate.

c) Blocking buffer. Buffers containing 1% w/v Bovine Serum Albumin (BSA), 0.5% w/v BSA, 1% w/v Dried Milk Powder (DMP) or 0.5% w/v DMP with or without the presence of Tween 20 (0.05% w/v) and, or PVP (2% w/v), were compared for their ability to prevent or reduce non-specific binding throughout the ELISA.

d) Washings. Washings with tap water, PBS and PBS-Tween were carried out and compared using the method described previously.

e) Incubations. To determine the optimum incubation times of first and second antibodies, at 37° C, all combinations of 2h, 1h and 0.5h were compared. Optimum incubation for coating of bacteria was then determined using the optimal 1st and 2nd antibody incubation periods.

f) ELISA design. Double antibody sandwich ELISA's (DAS) were compared with the indirect binding ELISA previously described. In DAS, microtitre plates were pre-coated with monoclonal antibody diluted 1:100 or with polyclonal antibody diluted 1:2000 in coating buffer, to "capture" the antigen (the bacteria). The routine ELISA protocol was then followed.

Diagnostic Assay

a) Plant samples. 1cm sections were cut from both the apical region and the lower part of the stem of an artificially infected potato plant (but with no observable Bacterial Wilt symptoms). Both pieces of tissue were macerated mechanically and resuspended in

0.05M carbonate buffer, pH 9.6, containing 0.2% of the antioxidant, sodium sulphite (1ml/g tissue), and 100μl added to relevant wells on a microtitre plate. The routine ELISA protocol was then followed.

b) Soil samples. 1g samples of artificially inoculated soil were suspended in 2ml coating buffer and shaken vigorously for 1 min. After allowing to settle for 2 min 100μl of the homogenate was removed and used in the routine ELISA. During testing two coating buffers were used: (i) 0.05M carbonate buffer, pH 9.6, and (ii) an amended coating buffer containing 8.3% polyvinyl pyrrolidine and 4% sodium cholate in 0.05M carbonate buffer, pH 9.6.

Results

Production of Antibodies

Both monoclonal and polyclonal antibodies have been raised to *P. solanacearum*. Figure 1 shows typical reactions of the polyclonal antibodies obtained with *P. solanacearum* and related bacteria, and also shows that the monoclonals produced to date show little more specificity than the polyclonals.

Optimisation of the ELISA

a) Plate type. Figure 2 shows the results obtained when different microtitre plates were tested. Both Griener (medium binding) and Nunc polysorp plates gave good results and were therefore used for all subsequent assays.

b) Coating buffer. The use of different buffers to coat the bacteria onto the plate made very little difference to the end results. 0.05M carbonate buffer, pH9.6, was chosen for further work, however, as slightly higher absorbance readings were obtained with no increase in background levels.

c) Blocking buffer. Dried milk powder (at 0.5%) was found to decrease background levels greater than Bovine Serum Albumin. When the dried milk powder was then used along with Tween 20 and polyvinyl pyrrolidone, non-specific binding was even further reduced without any detrimental effect on the sensitivity of the ELISA.

d) Washings. Washings done with PBS-Tween were found to be the most effective, giving lowest background readings with no loss in sensitivity.

e) Incubations. One hour incubations were found to be sufficient for all stages, with 30min sufficient for the second antibody.

f) ELISA design. Figure 3 shows the configuration of the three ELISA's which were

compared. Both DAS ELISA's were found to be less sensitive than the indirect ELISA, and so were not tested any further.

Diagnostic Assay

a) Plant samples. Using the routine ELISA, *P. solanacearum* was detected in artificially inoculated plants even when wilting was not apparent, (Elphinstone, unpublished).

b) Soil samples. When the routine ELISA was used for screening artificially inoculated soil samples, no bacteria were detected. If the bacteria were extracted from the soil particles and coated directly onto the plates using a buffer containing 8.3% polyvinyl pyrrolidine and 4% sodium cholate in 0.05M carbonate buffer, pH 9.6 (MacDonald, 1986), *P. solanacearum* could be detected in the soil down to levels as low as 1×10^4 cfu/ml, (bacterial levels as low as 1×10^2 cfu/ml were even detected in some assays).

Discussion

Several assays for the detection of *P. solanacearum* are currently being developed in laboratories worldwide. Many of these techniques, although excellent at detecting the bacteria, require sophisticated equipment and are difficult to perform in "the field". For example, the use of non-radioactive DNA probes to *P. solanacearum* and pathogen-specific DNA amplification by the Polymerase chain reaction (PCR), (Seal *et al.*, 1992).

In this paper we have detailed the development of an ELISA to detect *P. solanacearum*. The fact that this assay does not require sophisticated equipment, is relatively inexpensive and requires minimum of training, makes the assay ideal for use in developing countries where the disease is prevalent. The detailed ELISA is rapid and may be completed in as little as 3.5h (assuming it takes 15min for washing of plates and pipetting of reagents between incubations). It has also been shown to be very sensitive, routinely detecting bacterial levels of 1×10^4 cfu/ml, (although levels as low as 1×10^2 cfu/ml have been detected in some tests), making it ideal for the detection of such an economically important plant disease.

The ELISA may use either monoclonal or polyclonal antibodies, with monoclonals having the distinct advantage in that they generally show greater specificity and they may be produced as an unlimited supply. Throughout most of the experiments detailed in this paper, however, polyclonal antibodies were used. This was simply because they were readily at hand and the monoclonals available at the time showed little more specificity than the polyclonals. New protocols involving the use of the immunosuppressant

cyclophosphamide (Matthew & Sandrock, 1987) during immunisation of the mice, are currently underway in an attempt to improve specificity. Preliminary results have already shown an improvement and, if suitable, they may be used in future assays as standardised reagents. Such use of monoclonals can only improve the reliability of the assay.

Figure 1: Serological reactions of four polyclonal antibodies and one monoclonal antibody with a selection of bacteria isolates from the Rothamsted Culture Collection, as found by ELISA.

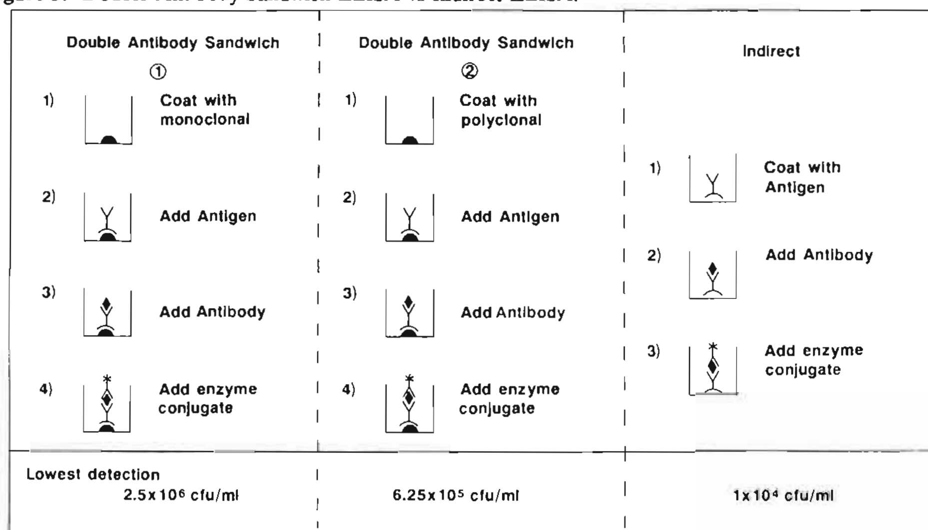
Bacterial isolate	Polyclonal				Monoclonal
	R230	R283	R303	R608	R038
A1 *P. cepacia*	-	+	-	+	-
A2 *P. cepacia*	-	-	-	-	-
R001 *P. syzygii*	-	+	+	+	+
R002 *P. syzygii*	+	+	+	-	+
R226 BDB	+	+	+	+	+
R230 BDB	+	+	-	-	-
R067 *X. fascians*	-	-	-	-	-
R036 *X. campestris*	-	-	-	-	-
R042 *P. fluorescens*	-	-	-	-	-
R043 *P. corrugata*	-	-	-	-	-
R011 *B. subtilis*	-	-	-	-	-
R111 *E. coli*	-	-	-	-	-
R137 *A. tumefaciens*	-	-	-	-	-
R038 *P. sol.* bv.1	+	+	+	-	+
R283 *P. sol.* bv.1	+	+	+	+	+
R303 *P. sol.* bv.2	+	-	+	+	+
R583 *P. sol.* bv.N2	+	+	+	+	+
R608 *P. sol.* bv.3	+	+	+	+	-
R279 *P. sol.* bv.4	-	+	+	+	+

BDB - Banana blood disease bacterium, *P. celebensis*. + detected, - not detected.

Figure 2: Evaluation of several commercially available microtitre plates.

Plate type	Background levels	Lowest detection level (cfu/ml)	Other factors	Rating
Griener, high	medium	1×10^7		moderate
Griener, medium	low	1×10^7		good
Nunc, maxisorp	high	5×10^7		moderate
Nunc, polysorp	low	5×10^7		good
Bioreba	medium	1×10^7	inconsistent	poor
Costar	very high	0		poor
Falcon, flexible	high	1×10^8	flexible plate	poor
Techne	high	1×10^8	flexible plate	poor

Figure 3: Double Antibody Sandwich ELISA vs Indirect ELISA.



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Antigenic differences in *Xanthomonas albilineans*, causal agent of leaf scald disease of sugar cane

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ABSTRACT

Two serotypes of *Xanthomonas albilineans* namely the Mascarene and African ones have been identified in Mauritius. The Mascarene one was more frequently encountered and 84% of the isolates characterized belonged to this serotype. Isolates from both serotypes produced similar symptoms in the field and had the same cultural characteristics, biochemical and physiological properties. Polyclonal antisera produced in rabbits were specific to each serotype in Ouchterlony's double diffusion test when using heat fixed bacterial cells as antigens. Protein profiles by polyacrylamide gel electrophoresis and fatty acid analyses of isolates did not reveal any differences between the two serotypes. However, different banding patterns were observed when the lipopolysaccharides (LPS) extracts from cultures of the two serotypes were compared on polyacrylamide gels. Transfer of the LPS to nitrocellulose membranes and probing with antisera showed that the LPS from each serotype reacted specifically with its homologous antiserum. It seems that the LPS is the antigenic component responsible for serotypic differences and this could prove useful in the production of monoclonal antibodies to each serotype.

KEYWORDS

Serotype, lipopolysaccharide, fatty acid, polyacrylamide gel electrophoresis.

INTRODUCTION

Leaf scald disease caused by *Xanthomonas albilineans* (Ashby) Dowson is an important disease of sugar cane world-wide. Recently, its incidence has increased dramatically in sugar cane plantations in Mauritius and the bacterium was found to be aerially transmitted causing infection through wounds on the leaf which was never reported before. Moreover, the bacterium was isolated from maize hybrids causing death of plants in extreme cases. These findings triggered studies in order to explain these observations. The bacterium was thus recovered in guttation droplets from infected sugar cane implying that the pathogen can be airborne (SORDIE & TOKESHI, 1986 ; AUTREY *et al.*, 1991a). In addition, field trials showed that isolates of *X. albilineans* differed in virulence but more importantly, a differential varietal response was present (AUTREY *et al.*, 1991b). Serological studies were also initiated so as to determine the relationship among the isolates. Previous work by ROTT *et al.* (1986) has shown that three serotypes of the pathogen exist, namely : Mascarene, African and Caribbean but only the first serotype was known to occur in Mauritius. In this paper, serological characterization of *X. albilineans* isolates from Mauritius, their biochemical properties as well as their fatty acid and protein profiles are reported. The antigenic components specific to two serotypes are discussed.

MATERIALS AND METHODS

Collection of isolates

Between 1984-1991, isolates were collected from maize and sugar cane during island wide surveys from foliar stripes (white pencil lines and yellow stripes) as well as from systemically infected stalks on Wilbrink medium (peptone, 5 g ; KH_2PO_4, 0.5 g ; $MgSO_4.7H_2O$, 0.25 g ; sucrose, 20 g and agar, 15 g per litre) and preserved at -20°C in Wilbrink broth supplemented with 20% glycerol.

Biochemical properties

Biochemical properties of 10 isolates from sugar cane and maize collected were determined using Biolog Microtitre Plates (Biolog GN Microplate, USA) containing 95 carbon sources. Bacterial cells from fresh cultures were prepared and plates inoculated according to the manufacturer's instructions. The optical density at 570 nm was measured using a Dynatech MR5000 reader.

Fatty acid profile

Ten isolates of *X. albilineans* collected between 1984-1991 from sugar cane and maize were sent to Dr D Stead, Central Science Laboratory, MAFF, Harpenden, UK for fatty acid profiling by gas chromatography and analysed using a Microbial Identification System software (Hewlett Packard).

Serological studies

Antisera obtained from P. Rott were tested against isolates of *X. albilineans* and new antisera were prepared as described by AUTREY *et al.*, (1989). Four isolates, two from foliar stripes in sugar cane (isolates 3095 and 3101), one from systemically infected sugar cane (3089) and one from maize (3086) were used for intravenous immunization in rabbits. Antisera were tested by Ouchterlony double diffusion in 0.6% purified agar in saline. Antigens consisted of whole live cells as well as heat treated ones (at 80 °C for 30 minutes) adjusted to a concentration of 1 x 10^9 cells/ml while antisera were used at a dilution of 1:4. A total of 140 isolates were characterized.

Extraction of whole cell protein

Cultures were grown till stationary phase in Wilbrink broth and 1.5 ml harvested by centrifugation at 1,100 g for 10 minutes in a microfuge. Proteins were extracted as described by AMES *et al.*, (1984).

Preparation of LPS

Lipopolysaccharides (LPS) were extracted from washed cells according to the method described by BRADBURY *et al.*, (1984) using 45% phenol solution and dialysed against distilled water for 48-72 h.

Electrophoresis

Polyacrylamide gel electrophoresis was carried out according to the discontinuous system of LAEMMLI (1970) using sodium dodecyl sulphate (SDS) for proteins and without for LPS. A 4.5% acrylamide stacking gel was used while running gels of 10% or 14% were adopted for protein and LPS respectively. Samples were mixed with an equal volume of sample buffer and 15 µl loaded in wells. Electrophoresis was carried out at 70 V until the bromophenol tracking dye in the sample bufffer entered the separating gel, after which the voltage was increased to 100 V.

Silver stain

Gels were stained by soaking overnight in fixative ethanol - glacial acetic acid - water (25:10:56), washed in distilled water for 1 h, incubated in 5 µg/ml dithiothreitol for 2 h and in 0.01% silver nitrate solution for another 2 h. After two quick rinses in distilled water, the gels were developed in 3% sodium carbonate solution containing 0.02% formaldehyde until bands appeared. The reaction was stopped by adding citric acid to a final concentration of 0.23 M.

Electrophoretic transfer

After separation by electrophoresis, the LPS were transferred to ECL nitrocellulose membrane (Amersham Ltd) by electroblotting at 200 mA for 2 h in a Hoeffer TE Transphor Unit using Towbin transfer buffer (TOWBIN *et al.*, 1979).

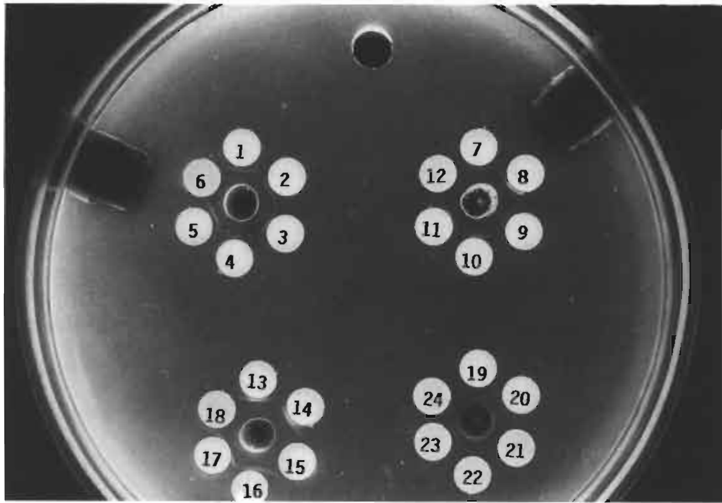
Immunoblotting of LPS

Membranes were blocked in PBS containing 0.05% Tween 20 and 5% dry non-fat milk (PBSTM) and transferred to 10 ml of antiserum prepared against isolates 3086 or 3101, diluted 1:500 in the same buffer. After overnight incubation, filters were washed and 10 ml of peroxidase-conjugated anti-rabbit IgG (Sigma Chemicals) diluted 1:5000 in PBSTM were added prior to incubation for 2 h at room temperature. Detections were carried out by using primarily the insoluble substrate 4-chloro-1-naphtol as described by HARLOW & LANE (1988). The chemiluminescent substrate Luminol (Amersham Ltd) was also used after immunoreaction and membranes were exposed to film for 30 seconds or longer.

RESULTS

Biochemical tests

All isolates had similar biochemical properties except for three carbon sources where a variable reaction was noted. Seventy eight carbon compounds were not utilised. All isolates tested oxidised N-acetyl-D-glucosamine, cellobiose, D-fructose, L-fructose, α-D-glucose, D-mannose, sucrose, methyl pyruvate, α-ketoglutaric acid, D,L-lactic acid, alaninamide, L-alanyl-glycine, L-glutamic acid and glycyl-L-glutamic acid. Variable reactions were observed with L-proline, L-serine and glucose-6-phosphate.



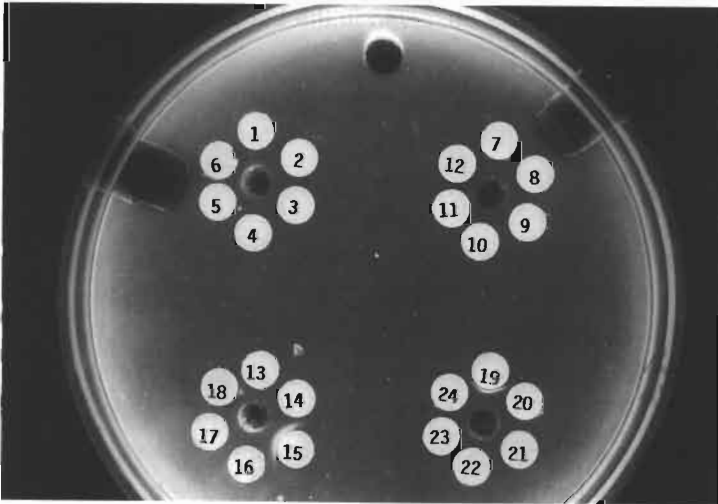


Plate 1. Immunodiffusion pattern of heat fixed cells of two serotypes of X.albilineans. Antiserum to Mascarene serotype (Top), African serotype (Bottom).

Centre wells: 25 µl of antiserum. Outer wells: 25 µl heat fixed antigen preparation. Wells 1,2,3,4,5,7,8,9,10,11,13,14,16,17,20: Mascarene serotype. Wells 15, 19: African serotype. Wells 6,12,18,21,22,23 and 24: saline control.

Fatty acid profile

The isolates included in the analysis belonged to a single cluster and formed part of the original *X. albilineans* profile in the library of the software package showing that no differences in the fatty acids were present.

Serological tests

Two distinct serotypes were found in double diffusion tests using the antisera obtained from P. Rott. They were the Mascarene and the African ones. Antisera prepared locally to isolates 3086 and 3089 were found to behave as the Mascarene serotype while 3095 and 3101 were typical of the African serotype. Specific precipitin lines were observed when bacterial cells were heat fixed (Plate 1) while with live cells, cross reactions were sometimes obtained. Out of 140 isolates characterised, 84% belonged to the Mascarene serotype while the remaining had African serotypic properties.

Electrophoresis and immunoenzymatic detection

A large number of protein bands were present in polyacrylamide gels. However, no differences were observed in the banding pattern among isolates.

LPS profile revealed differences among the isolates that corresponded to the Mascarene and African serotypes. More ladder like bands were present in the latter serotype than the former one (Plate 2).

Immunoenzymatic analysis of blotted LPS showed specific bands with homologous antigen and antiserum. Thus, LPS extracted from Mascarene serotype isolates reacted specifically with the antiserum against Mascarene isolates and no bands were formed with the antiserum of the African serotype (Plate 3). Similarly, the LPS from African serotype isolates reacted only with antiserum produced using African serotype isolates. These results show that the antigenic differences between the two serotypes are due to the LPS component.

Both the insoluble substrate 4-chloro-1-naphtol and the chemiluminescent one were effective in immunoenzymatic detection of LPS. However, for the latter substrate, immediate exposure to film is required due to the short period of light emission.

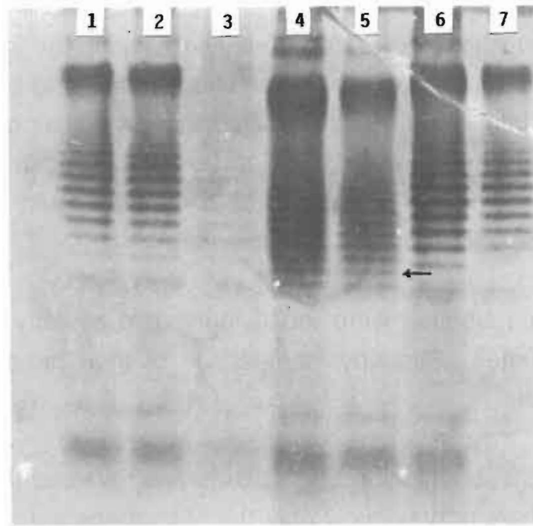


Plate 2.Polyacrylamide gel electrophoresis of LPS. Mascarene serotype : lanes 1,2,3,6,7; African serotype: lanes 4,5 with more ladder-like bands. Arrow indicates extra LPS band in African serotype isolates.

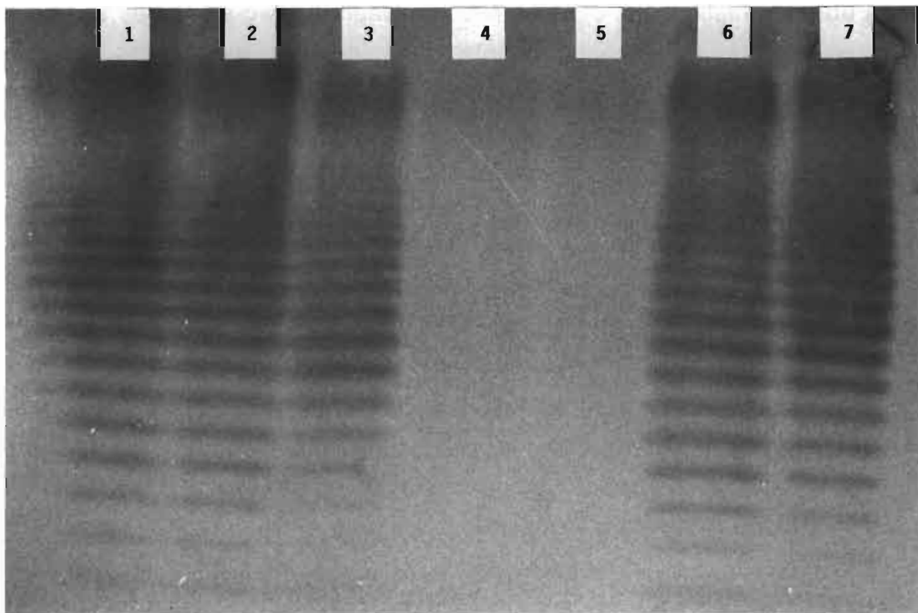


Plate 3. Immunoblot analysis of LPS with Mascarene serotype antiserum : lanes 1,2,3,6,7 (Mascarene serotype isolates) reacting specifically with homologous antiserum. No reaction in lanes 4 and 5 (African serotype isolates).

DISCUSSION

Laboratory investigations to detect variation among the leaf scald isolates in Mauritius were initiated as a result of observations in the field that were atypical of what were known about the disease. Biochemical properties of isolates collected from sugar cane and maize were found to be similar. However, three carbon compounds produced variable results, but no correlation could be established with the host, virulence or ability for aerial transmission.

The leaf scald isolates were indistinguishable by fatty acid analysis and formed a single cluster. Similarly, comparison of their protein profiles did not reveal any differences.

Serological characterization of *X. albilineans* revealed the existence of the African serotype whereas previously only the Mascarene serotype was known to occur. However, the recent epidemic of the disease could not be attributed to the occurrence of the African serotype as isolates of both serotypes have been found to be associated with the new phenomena.

Further investigations of differences between the two serotypes showed that they have distinct LPS profiles on polyacrylamide gels, the African one having more high molecular weight LPS than the Mascarene serotype. The differences were enhanced after transferring the LPS on membranes followed by immunoblotting. Specific reactions were obtained on incubation of the LPS with its homologous antiserum showing that the antigenic differences were due to the LPS component. LPS antigenic component has also been used in serogrouping of *E. carotovora* as well as many other enterobacterial species (DE BOER & MCNAUGHTON, 1987, DE BOER *et al.*, 1979).

The characterization of the antigenic differences between the two serotypes of *X. albilineans* would enable the production of specific antisera, such as monoclonal antibodies that would be useful in epidemiological studies. This approach is presently being investigated.

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A novel method for the validation of ELISA, based on immunomagnetic isolation of bacterial components and analysis with SDS-PAGE and Western blotting, demonstrated for ELISA detection of *Erwinia* spp. in potatoes

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Abstract

A method was developed to determine whether reactions in ELISA were caused by target antigens or by cross-reacting components present in plant extracts. *Erwinia carotovora* subsp. *atroseptica* and *Erwinia chrysanthemi*, both pathogens of potato, were chosen as target organisms.

Potato peel extracts were spiked with serial dilutions of bacterial suspensions from which the cells had been removed by filtration through a 0.22 μm filter. Magnetic beads, loaded with anti-target antibodies, were added to the extracts to enable selective concentration of soluble antigens. After an incubation period of one hour, the complexes were collected using a magnet and washed in a high molarity buffer (0.6 M NaCl). They were resuspended in 100 μl of a SDS sample buffer and boiled for five minutes. Samples were analysed with SDS-PAGE, followed by Western blotting using an anti-target serum.

The patterns on the Western blots for the *Erwinia* sp. could easily be distinguished from patterns of cross-reacting *Pseudomonas* sp. Potato peel extract components did not interfere with this method. The detection level was in the same range as that of DAS-ELISA.

Keywords

Cross-reacting bacteria, *Pseudomonas* spp., LPS

Introduction

Results of serological assays should be regularly checked for false-positive results caused by the presence of cross-reacting components in the sample, or by non-specific bindings during the test procedure. In ELISA, used for the detection of plant pathogenic bacteria, cell parts or soluble bacterial products are detected rather than whole bacterial cells, because cells are washed off during the wash procedure. Immunofluorescence cell-

staining and dilution plating methods are therefore no suitable methods for validation of ELISA, because they are only able to detect whole (living) bacterial cells.

A new method is presented that is able to analyse those bacterial products that are detected in ELISA. This method is demonstrated for detecting components of *Erwinia carotovora* subsp. *atroseptica* (Eca) and *Erwinia chrysanthemi* (Ech) in potato peel extracts.

Materials and Methods

Bacterial strains and growing conditions. In all experiments suspensions were used of Ech IPO strain nr. 502 and Eca IPO strain nr. 161, prepared from cultures grown for 24 h at 27 °C on trypticase soy agar (BBL) slants. The specificity of the verification method was evaluated using a selection of fluorescent *Pseudomonas* strains cross-reacting with Ech-antisera (VAN DER WOLF *et al.*, 1992) at concentrations of 10^8 cells/ml.

Preparation of soluble antigen samples. From bacterial suspensions of 10^9 cells/ml, cells were removed by subsequent centrifugation (10 min, 10 000 g) and filtration through a 0.22 μm filter. This 'stock-solution' was used for preparation of (serial) dilutions in phosphate buffered saline + 0.1% Tween 20 (PBST) and potato peel extracts, respectively.

Antiserum production. Antiserum 8276C and antiserum 8567G, raised against whole cells of Ech 502 and Eca 161, respectively, were used for conjugation of magnobeads. Antiserum Ram3KBC, produced against whole cells of Eca 161 and antiserum 9025C produced against total cell extracts of Ech 502 were used for Western blotting. The antisera were produced in rabbits, using the immunization procedure described by VRUGGINK & MAAS GEESTERANUS (1975).

ELISA. ELISA was carried out according to VAN DER WOLF & GUSSENHOVEN (1992).

Immunomagnetic concentration of bacterial antigens. A diagram of the verification procedure is shown in Fig. 1. Samples containing soluble antigens were transferred to a 24-wells tissue culture plate (0.5 ml/well).

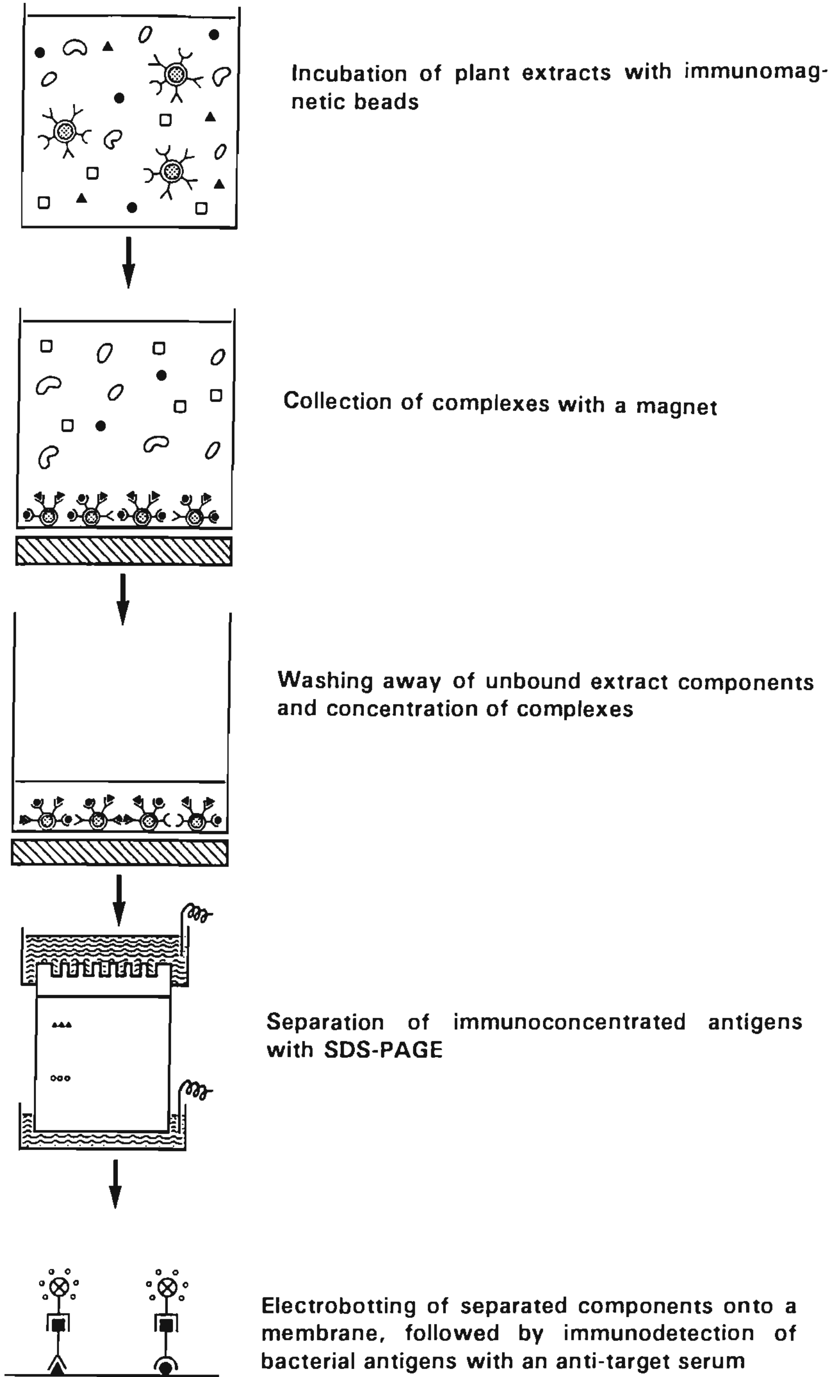

Fig. 1. Diagram of an evaluation method for ELISA-results

Para-magnetic beads (Advanced Magnetics Inc., 4100B), were conjugated with purified immunoglobulins according to the manufacturer's instructions. After dilution to 1 mg/ml in PBST, 0.15 ml beads were added to each sample and the mixture was incubated for 1 h at room temperature while shaking gently. Thereafter the complexes formed were washed four times for 10 min with PBST + 0.6 M NaCl, while shaking vigorously. Between the washing steps the magnetic beads were collected with a magnetic plate in order to enable removing of the washing buffer containing unbound material.

SDS-PAGE. Complexes were resuspended in 50 μl PBST and mixed with 50 μl of SDS-PAGE sample buffer. The suspension was boiled for 5 min and analysed with SDS-PAGE as described by LAEMMLI (1970) using 12.5% (w/v) separation gels and 3% (w/v) stacking gels.

Western blotting. Western blotting was carried out as described by FRANKEN *et al.* (1992).

Results and discussion

Using the verification procedure as described in Fig. 1, bacterial components equivalent to c. 10^6 cells/ml of the target bacteria could be detected (Fig. 2). This detection level was equal to the threshold level of a simultaneously performed double antibody sandwich ELISA using the same samples. The evaluation of the Eca immunoblot pattern was easier than of the Ech pattern, due to the high 'background' caused by strongly reacting lipopolysaccharide (LPS) O-chain molecules of Ech (VAN DER WOLF *et al.*, 1992).

Comparison of Fig. 2 with Fig. 3 clearly demonstrates the effect of the immunomagnetic isolation. Without immunomagnetic isolation, no target antigens could be detected, even not at concentrations of 10^8 cells/ml, due to the high background caused by extract components.

Fig. 4 illustrates that on the blot the total antigen pattern of Ech can easily be distinguished from that of fluorescent *Pseudomonas* species cross-reacting with Ech-antibodies (VAN DER WOLF *et al.*, 1992).

Immunomagnetic isolation and SDS-PAGE combined with Western blotting provides a specific method for analysis of those bacterial antigens in

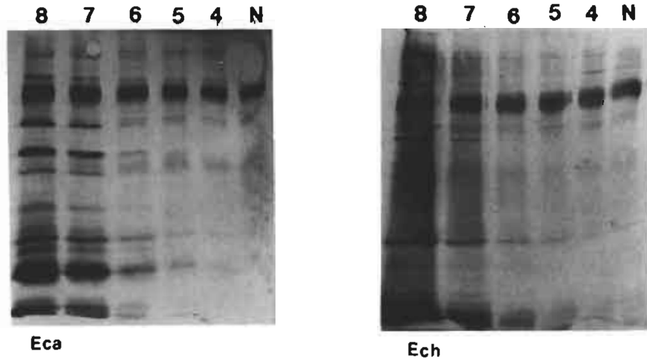

Fig. 2. Immunoblot of bacterial products of Eca and Ech, trapped from potato peel extracts with immunomagnetic beads, probed with a homologous antiserum.
Above the lanes, cell concentrations (^{10}log) are indicated. N = no antigens added.

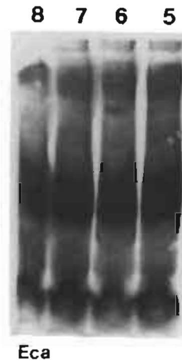

Fig. 3. Immunoblot of bacterial products of Eca spiked in potato peel extracts, probed with an anti Eca-serum. Above the lanes cell concentrations (^{10}log) are indicated.

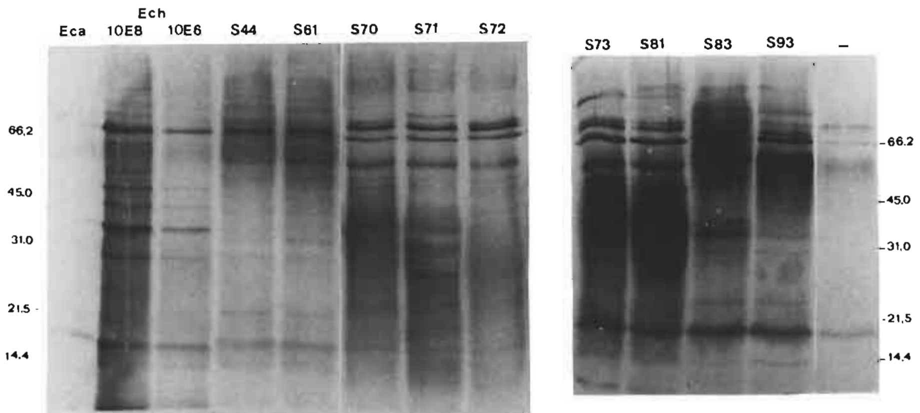

Fig. 4. Immunoblots of soluble antigens of Ech and of fluorescent *Pseudomonas* species, cross-reacting with Ech antibodies, probed with an anti Ech-serum. - = no antigens added.

plant extracts, that are also detected in ELISA.

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Detection of blood disease bacteria in infected banana plants using "monospecific" antibodies

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Summary

A close serological relationship was observed between blood disease bacteria (BDB) and Pseudomonas solanacearum (Pso) using traditional antisera against whole cells of both bacteria.

A selected protein band of BDB was used as antigen to produce antibodies from rabbit. These newly developed antibodies reacted specifically with all virulent strains of BDB in agglutination tests and colony blot tests, but no or only very weak reactions were found with Pso and other bacterial species.

The specificity of the antibodies was confirmed in studies with plant extracts. BDB could be detected in all infected plants at different time intervals (3 - 33 days) after inoculation, whereas plants inoculated with Pso race 2 never showed a positive reaction.

Introduction

Blood disease on banana was first confined to Sulawesi, where it causes a systemic and lethal wilt of banana and plantain (7). Unfortunately, the disease recently spread to other islands in Indonesia, e.g. Java, Moluccas, Kalimantan, Sumatera, Irian Jaya, and is considered the most serious threat to banana production in Indonesia, particularly in Sulawesi (1, 8).

Until now, no banana cultivars were found resistant towards the disease; several plant species were even found susceptible, like *Heliconia* spp, *Canna indica*, *Solanum nigrum*, *Asclepias currassiva* and *Strelitzia vaginae* (1).
On the other hand, moko disease of banana and plantain caused by *Pso* race 2 is

endemic in Central and South America (3,6) and was found also in Asia (10). The symptoms of both diseases are relatively similar : systemic, lethal wilt and internal discolouration of the stem and fruit.

Serological methods were used for detection and identification of the pathogens. A close serological relationship was observed between the BDB and *Pso* using traditional antisera which were produced against the bacterial cells. Therefore, in this study we examined the specifity of newly developed antibodies to both bacteria using micro-agglutination and colony-blot tests.

Materials and Methods

Origin and growth of bacteria

Isolates of BDB were mainly obtained from diseased banana plants in Sulawesi, Java, Moluccas and Kalimantan ; a few isolates were provided by Dr. Eden-Green, Rothamsted Experiment Station, Herts, England.

Isolates of *Pso* were obtained from our collection (GSPB) and from ICMP, New Zealand ; NCPPB, and Rothamsted Experiment Station, England (Table 1). All bacteria were grown and maintained in Kelman's tetrazolium medium, CPG or NGA medium, and incubated at 29°C for 2-3 days after transfer.

Preparation of antisera

Four different antisera were compared. Antiserum AS389 was raised from rabbit directed against BDB (isolate PO2). Antiserum AS257 was raised from rabbit against *Pso* race 2 (isolate 1508) and antiserum AS 4990 was raised from goat against a mixture of races 1, 2, and 3 of *Pso*. The latter two antisera were obtained from Dr. I. Krämer, Bundesanstalt für Züchtungsforschung, Aschersleben, Germany. Antiserum SAK was raised from rabbit against a selected protein band of BDB. The proteins of BDB were seperated by SDS-PAGE and absorbed with rabbit antiserum AS389 using Western blotting (2).

Agglutination test with whole cells

Agglutinations with bacterial cells on slides were performed as described by Niepold and Huber (9).

Colony-blot test

Pure cultures or bacteria from diseased banana were serially diluted in 0.01 M $MgSO_4$ and then streaked on CPG medium. The plates were incubated at 29°C for 2-3 days. Plates with 30-50 bacterial colonies were selected for use in the test. A nitrocellulose (= NC) filter was placed on to the bacterial colonies and pressed with a glass spatula. The removed first NC filter and a second filter were pressed together between two glass Petri dishes. The second NC filter was incubated 5 min in sodium dodecyl sulfate (SDS) solution adsorbed to a membran filter and was washed with

Table 1. Reaction of antibodies against different isolates of BDB and *P. solanacearum* in micro-agglutination tests

No. of Isolate (GSPB No.)	Infected Cultivars	Geographic origin and Source	Reaction with AS389	AS257	AS4990	SAK	K
Blood Disease Bacteria							
P02 (1845)	ABB	S.Sulawesi	+++	++	±	++	-
T334 (1790)	AAA	W.Java, E.Green	+++	++	++	++	-
H22a (1891)	AAA	W.Java	+++	++	nt	++	-
T440 (1793)	ABB	N.Sulawesi, E.Green	++	++	nt	+	-
H02 (1843)	AAB	S.Sulawesi	++	+	++	++	-
MO1b (1896)	ABB	Moluccas	++	++	nt	++	-
H02 (1843)	ABB	S.Sulawesi	++	+	+	++	-
MO1a (1895)	ABB	Moluccas	+++	+	+	+	-
H11 (1890)	ABB	S.Sulawesi	+	+++	+	±	-
H18 (1854)	ABB	S.Sulawesi	++	++	+++	++	-
H22b (1892)	AAA	W.Java	+++	++	++	++	-
H03 (1844)	ABB	S.Sulawesi	+++	+	+	++	-
B2e (2138)	ABB	E.Kalimantan	+++	++	++	++	-
B2i (2137)	ABB	E.Kalimantan	++	++	++	+	-
P. solanacearum							
523	Bean	Mauritius, GSPB	++	+	+++	-	-
2015	Tomato	USA, RCC	+	+	++	-	-
1959	Ginger	Hawaii, NCPPB	+	+	++	-	-
2014	Tobacco	Australia, NCPPB	+	+	+++	-	-
2111	Capsicum	Indonesia, RCC	+++	nt	++	-	-
2116	Ginger	,, ,,	+++	nt	+++	-	-
2118	Ricinus	,, ,,	+++	nt	++	-	-
2124	Potato	,, ,,	+++	nt	+++	-	-
1508	ABB	Costa Rica, GSPB	++	++	+++	-	-
1510	AAB	,, ,,	-	+++	+++	-	-
1512	AAA	Costa Rica, RCC	+++	+++	+++	-	-
2014	AAA	,, ,,	-	+	+++	-	-
2123	AAA	Philippines, RCC	++	nt	++	-	-
2112	AAA	Colombia, RCC	+	nt	+++	-	-
2115	Heliconia	Costa Rica, RCC	+++	nt	+++	-	-
2134	Strelitzia	Taiwan,RCC	+++	nt	+++	-	-
2125	ABB	Peru, RCC	++	nt	++	-	-
2130	Mulberry	China, RCC	+++	nt	+++	-	-
2132	Olive	,, ,,	+++	nt	+++	-	-
P. sizygii							
2088	Clove	Indonesia, RCC	+++	+	+++	-	-
2089	,,	,, ,,	+++	++	+++	-	-
2090	,,	,, ,,	+++	++	+++	-	-
X.c. pv. celebensis							
1782	Banana	Indonesia, ICMP	-	-	-	-	-
1630	Banana	,, , NCPPB	-	-	±	-	-

nt: not tested. GSPB: Göttinger Sammlung Phytopathogener Bakterien, Germany. ICMP: Int. Collection of Microorganisms from Plants, New Zealand. NCPPB: National Collection of Plant Pathogenic Bacteria, England. RCC: Rothamsted Culture Collection, England.

H_2O three times and once with TN buffer. The filters were blocked with 3% BSA-TN for 2 h and incubated with SAK overnight. The blots were washed with TNT (10 mM Tris HCl, 150 mM NaCl, 1 % Triton x 100, pH 8.0) 3 x 10 min and TN (10 mM Tris HCl, 150 mM NaCl, pH 8,0) 1 x 10 min. Anti-rabbit IgG coupled with alkaline phosphatase (1 : 1000 in 1 % BSA-TN) used as secondary antibody was incubated with the blots for 2 h. Then, the blots were washed with 1% BSA-TN 3 x 10 min and TN 1 x 10 min. Staining of the blots was achieved by Fast Red.

Results

Micro-agglutination test

The results of testing the antisera against BDB, *Pso* and other bacteria are summarized in Table 1. Not only AS389, but also AS257, and AS 4990 showed cross reaction to BDB and *Pso*. The antisera also reacted with *P. sizygii*, which causes Sumatera wilt disease on clove in Indonesia.

The contrary was observed with monospesific antibodies (SAK) raised against the selected protein band of BDB. These reacted only with BDB isolates, except for a weakly reaction with avirulent BDB isolate (H11), and did not react with isolates of *Pso* or other bacteria.

Colony-blot test

The test was used to detect BDB not only from pure bacterial cultures but also from infected banana plants. The method is more sensitive than the agglutination test. Table 2 shows that up to a dilution of 1 : 750 the specific antibodies reacted strongly with BDB. Almost all isolates of BDB from pure culture reacted very well with the specific Abs, only isolate HO8 (weakly virulent) reacted weakly positive, whereas all isolates of *Pso* were found negative except one which exhibited a weak reaction (Table 3).

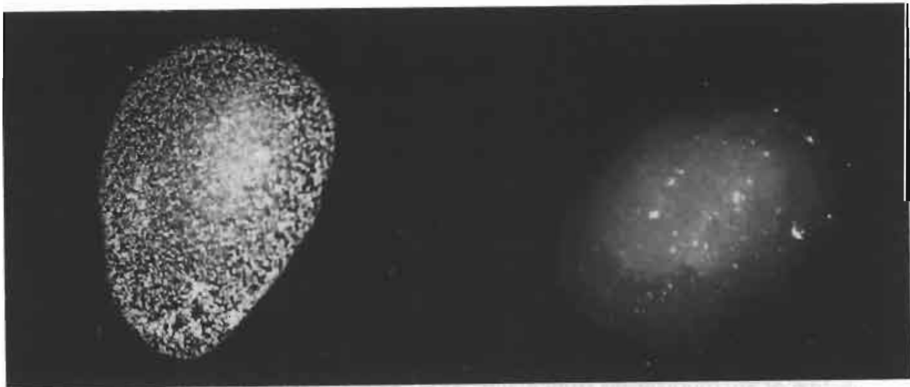

Fig. 1. Agglutination of BDB by monospecific antibodies (left) and negative reaction of *P. solanacearum* (right)

Table 2. Determination of dilution of specific antibodies against BDB (isolate P02)

Titer of antibody	reaction
undiluted	++
1 : 250	++
1 : 500	++
1 : 700	++
1 : 1000	+
1 : 1250	±
1 : 1500	±
control A (without SAK and conjugate)	-
Control B (without conjugate)	-

Table 3. Reaction of the antibodies against pure cultures BDB and *Pso* in colony-blot tests

Reaction with SAK (1:750)			
A.BDB str.		B.*Pso* str.	
1.PO2	++	1. 1511	-
2.H22a	++	2. 1115	-
3.HO3	++	3. 6782	±
4.MO1a	++	4. 1508	-
5.PO3	++	5. 1579	-
6.HO2	++	6. 1513	-
7.T334	++	7. 2014	-
8.T340	++	8. 1509	-
9.HO8a	+	9. 1510	-
		10. 1511	-
		11. 1512	-
		12. 2015	-

Figure 2 shows the amount of BDB colonies detected on NC filter in isolations from banana plants at different time intervals after inoculation. On agar medium higher numbers of colonies were counted because other bacterial contaminants were includes. Also isolations from Pso infected banana plants resulted in high numbers of bacterial colonies on agar medium, whereas on NC filters no serologically positive colonies were seen.

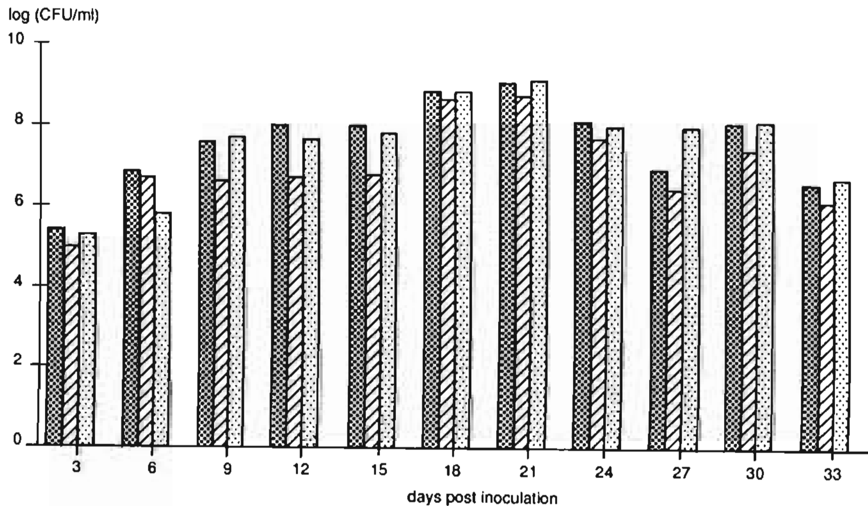

Fig. 2 The ability of the antibodies to detect BDB isolated from artificially inoculated banana plants

Discussion

The studies demonstrated the specificity of antibodies (SAK) for the differentation of BDB from *Pso* and other bacterial species. In the colony-blot tests, the antibodies could detect BDB from artificially inoculated banana plants with high sensitivity. Thus BDB were detected in plants at all stages between 3 - 33 days after inoculation, although symptoms did not appear before approx. 2 weeks after inoculation. The detection level of the BDB ranged between 10^4 - 10^9 cells/ml in these experiments.

The results indicate that the antibodies can detect BDB in plants during latent infection. It has not been investigated, whether it is possible to detect BDB from naturally infected plants or from insects vectors with this method. In comparison with ELISA or IF, the colony-blot test appears more advantageous, because this method does not require expensive laboratory equipment like spektrophotometer or fluorescence microscopy. Althought the BDB of banana has not yet been fully characterized, several properties distinguish the bacterium from Pso, which also cause wilt disease on banana and plantain.

Also by metabolic capabilities (Biolog System) the two pathogens can be distinguished (11). Eden-Green et. al (1988) found that BDB can neither reduce nitrate nor hydrolyse gelatin. Other characteristics of BDB are : non fluorescent; poly-ß-hydroxybutyrate positive; mucoid, small round colonies with a red center on tetrazolium medium ; oxidase positive; HR positive (1,5).

Numerical analysis of 78 phenotypic characters showed that BDB forms an intermediate cluster between Pso biovar 1 and *P. syzigii* (4).

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Identification of *Erwinia amylovora* with monoclonal antibodies

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ABSTRACT

A panel of hybridomas secreting monoclonal antibodies (MAb) specific for *Erwinia amylovora* were produced by fusing NS-1 murine myeloma cells with the splenocytes of a BALB/c mouse immunised with heat-killed cells of *E. amylovora*. Antibody specificity was determined using indirect ELISA. *E. amylovora* was distinguished from other plant pathogenic bacteria from the genera *Agrobacterium*, *Clavibacter*, *Pseudomonas*, *Xanthomonas* and also species of *Bacillus* and *Escherichia*. MAb 36-2-3, 36-2-4 and 36-2-5 did not cross-react with *E. herbicola* and species of the soft-rot *Erwinia*, i.e. *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroseptica* and *E. chrysanthemi*. A high proportion of the antibodies cross-reacted with *E. herbicola* suggesting this bacterium probably shares more common antigenic epitopes with *E. amylovora*. Different reactivity patterns were obtained with the panel of antibodies against different strains of *E. amylovora* indicating strain diversity amongst *E. amylovora*. Selected MAb were of immunoglobulin isotypes IgG1, IgG2a and IgG2b. The molecular weights as determined by SDS-PAGE ranged from 10604 to 38600 daltons.

KEY WORDS: Monoclonal antibodies, *Erwinia amylovora*, ELISA.

INTRODUCTION

Fire blight of apple and pear, caused by *Erwinia amylovora* (Burrill) Winslow *et al.*, is a disease of international importance. Early detection of the bacterium on plants or fruit will assist prevention of disease spread. Methods which speed up the detection and identification process are invariably useful in monitoring fire blight outbreaks. The advent of hybridoma technology (Kohler and Milstein 1975) has widened the scope for improving the specificity of immunoassays. Monoclonal antibodies (MAb) have been shown to offer specific detection of *E. amylovora* (Lin

et al 1987, McLaughlin *et al.* 1989). This paper reports a modified method for production of MAb to *E. amylovora* and preliminary results on evaluation of antibody specificity.

MATERIALS AND METHODS

Antigen preparation. Bacterial strains used in this investigation were obtained from the International Collection of Micro-organisms from Plants, Department of Scientific and Industrial Research, Auckland, New Zealand. *E. amylovora* strain number PDDCC 1540 (Ea1540) was used for immunisation. Bacterial strains were cultured on nutrient agar at 27°C for up to 48 h. The cultures were washed off the agar plates with phosphate-buffered saline (PBS) (pH 7.4), and centrifuged 3 times at 13 000 g for 10 min. The turbidity of the cell suspension was measured at wavelength 540nm and the cell numbers enumerated. Heat-killed antigens were prepared by heating the suspension in a water bath at 100°C for 90 min. The suspensions were diluted in PBS to 10^8 cells/ml for immunisation and ELISA screening.

Production of monoclonal antibody. BALB/c mice were injected peritoneally three times every two weeks with heat-killed cells of Ea1540. Fusion with murine myeloma cells NS-1 was carried out five days after the final injection. Spleen cells, at $5x10^7$ cells in Iscove's Modified Dulbecco's Medium (IMDM) (Gibco BRL) were fused with $2x10^7$ myeloma cells in polyethylene glycol (MW 4000). The cells were washed and resuspended in IMDM amended with 10% foetal calf serum, 0.1 mM hypoxanthine, 0.0004 mM aminopterin and 0.016 mM thymidine. The mixture was distributed into two 24-well culture plates (Nunc, Denmark) containing an overnight culture of macrophage at $2x10^4$ cells/well.

Screening by ELISA. Culture supernatants were screened using indirect ELISA (Clark and Adams 1978) with some modifications. The reagents used were 200-220 ul/well. Plates were washed twice with tap water between stages unless specified. The antigens were incubated overnight at 5°C in 96-well microtitre (Nunc Immuno) plates. The plates were then incubated for 120 min at 27°C with conjugate buffer amended with 1% bovine serum albumin. Culture supernatants were incubated for 200 min at 27°C. Anti-mouse IgG-alkaline phosphatase conjugate (Kirkegaard & Perry Lab) at dilution 1:1000 was incubated for 90 min at 27°C. The plates were then washed twice in tap water, once with PBS-Tween and finally with tap water. Then *p*-nitrophenyl-phosphate (Sigma) was added at 1 mg/ml and the reactions allowed to occur at room temperatures.

Specificity of MAb. Culture supernatants collected from the macrocultures were screened against bacterial species and antigenic preparations as listed in Table 1. Strains of *E. amylovora* were also examined for likely serological diversity among this species.

Antibody isotype determination and SDS-PAGE. Selected macrocultures were subcloned and the antibody isotypes of the culture supernatants were determined using a Mouse MonoAb-ID EIA Kit (Zymed Lab., California). MAb were purified by precipitating with saturated $(NH_4)_2SO_4$. The molecular weights were determined by vertical gel electrophoresis in a Protean II xi electrophoresis cell (Bio-Rad, California).

RESULTS AND DISCUSSION

The hybridomas produced antibodies which did not cross-react with bacteria from other genera, e.g. *Agrobacterium*, *Clavibacter*, *Pseudomonas*, *Xanthomonas*, and *Bacillus*. There were generally more cross-reactions with *Escherichia coli* as it is more closely related to the Erwinias than with other phytopathogenic bacteria (Table 1). Cross-reactions with other bacteria using antibodies raised against sonicated cells has been reported (Lin *et al.* 1987). In this study, heat-killed cells were shown to be useful in production of antibodies which recognise common epitopes in *Erwinia* spp. In addition, these antibodies did not react with formaldehyde-fixed and extracellular polysaccharide preparations of Ea1540.

Antibodies produced by macroculture 36 were species-specific and did not cross-react with the soft-rot *Erwinia* spp., such as *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* and *E. chrysanthemi.* However, antibodies produced by macrocultures 11 and 18 reacted strongly with all *Erwinia* spp. suggesting recognition of common epitopes among *Erwinia* spp. A higher proportion of the macrocultures cross-reacted with *E. herbicola* than with other *Erwinia* spp. This indicates *E. herbicola* is serologically more closely related to *E. amylovora.* Since *E. herbicola* is a common saprophyte on apples and pears and has the potential to be used as a biological control agent, it is essential that an antibody that will distinguish the saprophytes from the pathogen is used in the detection of *E. amylovora*. The reactivities of selected MAb with *E. amylovora* and *E. herbicola* are summarised in Table 2. Specific MAb which did not cross-react with *E. herbicola* were 36-2-2, 36-2-4 and 36-2-5 (Table 2).

Table 1. Reactivity of selected hybridoma culture supernatants and polyclonal antibodies with various heat-killed bacteria at 10^8 CFU/ml and antigenic preparations of Ea1540 in an indirect enzyme-linked immunosorbent assay.

Macro-culture number	Species of bacteria												
	Ea	Eh	Eco	Ecc	Eca	Ea F	Ea EPS	Pf	Xc	Bs	Pv	Cm	At
Polyclonal antibodies	9	7	9	6	4	1	2	1	2	1	-	1	1
7	9	6	7	3	2	1	1	-	-	-	-	-	-
11	9	9	5	2	1	1	2	-	-	-	-	-	-
18	9	3	4	4	2	1	1	-	-	-	-	-	-
16	5	1	2	3	2	-	-	-	-	-	-	-	-
32	5	2	1	-	-	-	1	-	-	-	-	-	-
28	4	-	-	1	-	1	1	-	-	-	-	-	-
5	3	1	2	2	-	1	-	-	-	-	-	-	-
19	9	6	4	-	1	1	2	-	-	-	-	-	-
13	9	9	-	-	-	-	1	-	-	-	-	-	-
9	9	4	-	-	-	1	2	-	-	-	-	-	-
34	7	1	-	-	-	-	1	-	-	-	-	-	-
36	6	-	-	1	-	-	-	-	-	-	-	-	-
17	5	1	1	-	-	-	-	-	-	-	-	-	-
4	5	6	1	1	1	1	1	-	-	-	-	-	-
33	4	-	-	-	-	-	-	-	-	-	-	-	-
26	2	-	-	-	-	-	-	-	-	-	-	-	-
12	2	-	-	-	-	-	-	-	-	-	-	-	-
31	1	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-	-
Mean reaction (47 macro-cultures)	5	2	2	1	-	1	-	-	-	-	-	-	-

Ranking of ELISA ratios (Absorbance 405nm / Absorbance lowest 405nm) % : 0-10 (-) 11-20 (1) 21-30 (2) 31-40 (3) 41-50 (4) 51-60 (5) 61-70 (6) 71-80 (7) 81-90 (8) 91-100 (9)

Ea = *E. amylovora* 1540, Eh = *E. herbicola* 272a, Ecc = *E. carotovora* pv. *carotovora* 39,
Eca = *E. c.* pv. *atroseptica* 549, Ea F = *E. amylovora* 1540 formaldehyde fixed,
Ea EPS = *E. amylovora* 1540 extracellular polysaccharide, Eco = *Escherichia coli*,
Xc = *Xanthomonas campestris* pv. *versicatoria* 65a, At = *Agrobacterium tumefaciens* 5856a,
Pf = *Pseudomonas fluorescens* 3512, Cm = *Clavibacter michiganensis* pv. *michiganensis* 2558,
Bs = *Bacillus subtilis* 368, Pv = *Pseudomonas viridiflava* 2848

Table 2. Isotypes of antibody, molecular weights determined by SDS-PAGE and ELISA reactivities of MAb with heat-killed cells of *E. amylovora* Ea1540 and *E. herbicola* 272a at 10^8 CFU/ml.

Macro-culture number	Monoclonal antibody	Antibody isotype	Mol. wt	ELISA reactions at 405nm *E. amylovora* Ea1540	*E. herbicola* 272a
Polyclonal antibody				2.12	2.04
4	4-2-1	IgG2a	38600	2.26	1.94
9	9-2-1	IgG1	-	0.30	0.08
17	17-1-1	*	38153	1.80	0.83
	17-1-2	IgG2b	*	2.00	0.89
18	18-1-5	IgG2a	10604	1.29	1.25
36	36-2-2	*	*	1.40	0.14
	36-2-4	*	19000	1.20	0.13
	36-2-5	*	*	1.50	0.15

* Not tested - No reaction with denatured antigenic components

The antibodies listed in Table 2 belonged to immunoglobulin subclasses IgG1, IgG2a and IgG2b. The molecular weights as determined by SDS-PAGE were in the range of 38600, 38100 and 10600 daltons. One antibody specific to *E. amylovora*, 36-2-4 had a molecular weight of 19000 daltons.

Different reactivity patterns were obtained with the panel of antibodies against 8 strains of *E. amylovora* (Table 3). The initial findings indicate recognition of strain-specific epitopes in *E. amylovora*. However, Lin *et al* (1987) suggested a high degree of conservation in antigenicity in their isolates. Selected MAb need to be screened against more *E. amylovora* strains to clearly establish the serological diversity of this organism.

This method of antibody production and screening has allowed a more manageable schedule compared with screening the fusion mixture in five 96-well culture plates. Subcloning can be carried out subsequently on targeted macrocultures. The method has allowed easy selection and grouping of antibodies according to their specificity and provided an overall pattern of serological relationships of the isolates.

Table 3. ELISA reactivity of selected hybridoma culture supernatants and polyclonal antibodies with various heat-killed strains of *E. amylovora* at 10^8 CFU/ml.

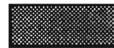
Macro-culture number	Strains of *E. amylovora*							
	1540	1493	1495	8861	1501	1507	9144	1440
Polyclonal antibodies	81-100	81-100	81-100	81-100	81-100	81-100	81-100	81-100
11	81-100	81-100	81-100	81-100	81-100	81-100	61-80	0-20
10, 13	81-100	81-100	81-100	81-100	81-100	81-100	0-20	0-20
7, 19	61-80	41-60	61-80	61-80	61-80	61-80	61-80	0-20
9	61-80	41-60	61-80	41-60	61-80	41-60	0-20	0-20
32	41-60	21-40	41-60	41-60	41-60	81-100	81-100	81-100
27	41-60	21-40	41-60	41-60	21-40	81-100	21-40	41-60
18	41-60	21-40	41-60	41-60	41-60	41-60	21-40	21-40
5	21-40	0-20	21-40	21-40	21-40	41-60	61-80	61-80
40	21-40	0-20	21-40	21-40	41-60	0-20	0-20	61-80
20, 23	21-40	0-20	21-40	21-40	21-40	0-20	61-80	0-20
44	21-40	0-20	21-40	21-40	21-40	21-40	81-100	21-40

Ranking of ELISA ratios (Absorbance 405nm / Absorbance lowest 405nm) % :

0-20

21-40

41-60

61-80 

81-100

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Localization of phytopathogenic bacterial antigens in plant tissue sections by immunoprinting ELISA using biotinilated monoclonal antibodies

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ABSTRACT

A new method named "Immunoprinting-ELISA" (IP-ELISA) has been used to localize Erwinia carotovora subsp. atroseptica and Xylophilus ampelinus bacterial antigens in tissue sections of stems of grapevine and potato plants, respectively, using monoclonal antibodies (MCA). Fresh and smooth sections of stems were pressed onto a nitrocellulose membrane and the direct tissue blotting was incubated with bovine serum albumin. The membranes were washed and incubated with MCA biotin labelled (IP-ELISA-direct). After washing membranes, streptavidin-alkaline phosphatase linked were added and incubated. A precipiting substrate for alkaline phosphatase was added and reaction was stopped after a few minutes by washing with distilled water. Final coloured spots or areas were observed using a low power magnification on a stereo binocular. The reaction has also been accomplished using MCA and goat anti mouse-alkaline phosphatase (IP-ELISA-indirect). Correlation was observed between positive IP-ELISA (direct or indirect) and isolation of the bacterium, indirect immunofluorescence or DAS-ELISA.

KEYWORDS

Immunoprinting, ELISA, detection, monoclonal antibodies, Erwinia carotovora subsp. carotovora, Xylophilus ampelinus, potato, grapevine.

INTRODUCTION

Immunological procedures are commonly utilized in detection of plant pathogenic bacteria and improved detection procedures are to suit specific applications in detection of bacterial antigens. Tissue printing on nitrocellulose paper has been used to study the development of soybean seed coats (Cassab and Varner, 1987) and to detect virus and mycoplasmas (Lin *et al.*, 1990 ; Cambra *et al.*, 1991).

The usefulness of the new immunoprinting-ELISA (IP-ELISA) method to detect plant pathogenic bacteria in fresh plant tissue sections is presented.

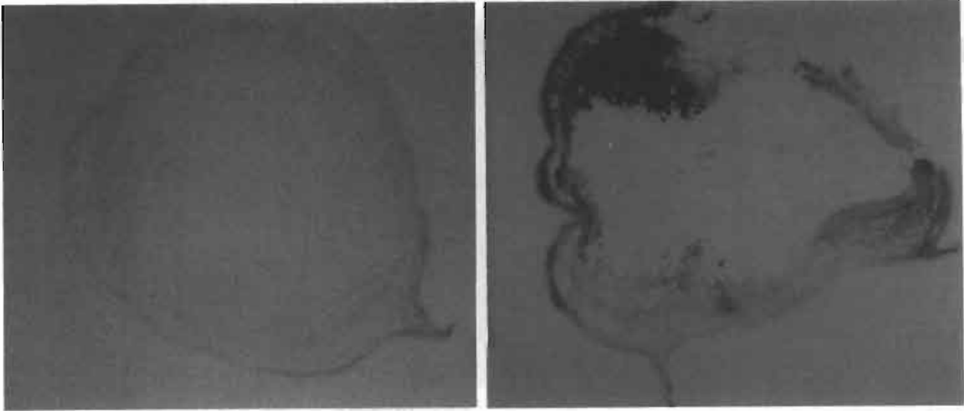

Fig. 1.

Immunoprinting: sections of healthy and diseased plants.

MATERIALS AND METHODS

The following technique has been used to detect Erwinia carotovora subsp. atroseptica (Eca) in artificially infected grapevines. Specific monoclonal antibodies obtained at IVIA were used in both experiments.

Fresh stem sections were pressed onto a nitrocellulose 0,45µ membrane until leaving a printing mark. It includes known sources and controls : healthy and infected plants. The tissue blottings were dried for a few minutes and incubated for 1 h at room temperature in 1% bovine serum albumin (or overnight at 4°c). This was followed by incubation for 1-2 h at room temperature in 0.1 - 0.2 µg/ml biotin labelled specific monoclonal antibody in PBS (IP-ELISA-direct) or with the specific monoclonal antibody or ascitic fluid (IP-ELISA-indirect). After three washings (x5 minutes) with gentle agitation in PBS-Tween two procedures were used. (i) IP-ELISA-direct : a solution of streptavidin-alkaline phosphatase conjugate was added and incubated for 30 minutes at room temperature or (ii) IP-ELISA-indirect : goat anti-mouse immunoglobulins alkaline phosphatase conjugate (0.1 µg/ml), were added and incubated for 1-2 h at room temperature, followed by three washings. The precipiting substrate for alkaline phosphatase (substrate buffer) was : (0.1 M Tris-ClH + 0.1 M NaCl + 5 mM MgCl ; pH 9.5). 0.33 mg Nitro Blue Tetrazolium (NBT)/ml substrate buffer + 0.175 mg Bromo Chloro-Indolyl Phosphate (BCIP)/ml substrate buffer were added followed by incubation for several minutes at room temperature. When colour change was observed the reaction was stopped by washing with distilled water. The coloured spots or areas were observed using a low power magnification on a stereo microscope.

Comparison of Eca isolation on CVP modified medium (Perombelon *et al.*, 1991) and Eca detection by DAS-ELISA conventional (Sanchez-Vizcaino y Cambra, 1987) and IP-ELISA was performed in potato plants artificially inoculated with Eca at the stem base. MCA 4G4 was used for DAS-ELISA and IP-ELISA (Alarcón *et al.*, 1992). Sampling points were designated 1 (root) to 8 (upper shoot).

Comparison of external symptoms, DAS-ELISA conventional and IP-ELISA for X. ampelinus detection was performed in grapevine plants artificially inoculated with this bacterium. A mixture of MCAs 5C, 8C and 5G were used (Cambra *et al.*, 1989) for DAS-ELISA and IP-ELISA.

RESULTS AND DISCUSSION

Table 1 shows the positive Eca samples detected by the different techniques vs the total number of potato samples. A high correlation between the different techniques was observed. IP-ELISA could detect the bacterial antigens in the highest number of samples.

Table 1

Comparison of isolation and conventional DAS-ELISA with immunoprinting-ELISA for detection of Erwinia carotovora subsp. atroseptica on potato.

	Methods		
Potato sampling points	Isolation	DAS-ELISA *	IP-ELISA *
1	3/5	3/5	4/5
2	3/5	3/5	2/5
3	7/8	7/8	8/8
4	9/9	9/9	9/9
5	9/10	8/10	9/10
6	2/10	2/10	2/10
7	0/10	1/10	2/10
8	0/9	0/9	1/9
TOTAL	33/66	33/66	37/66

* Fractions indicated the positive samples vs the total number of potato samples.

Table 2

Comparison of symptomatology and DAS-ELISA conventional with IP-ELISA to detect Xylophilus ampelinus on grapevine.

Symptomatology	DAS-ELISA	IP-ELISA
Positives samples 21	21	21
Negative samples 13	6*	5*

* The remaining samples, up to 13, were positive in DAS-ELISA or in IP-ELISA.

Table 2 shows the positive and negative X. ampelinus samples obtained by the different techniques on the 34 plants analysed. A high correlation was observed between DAS-ELISA and immunoprinting but not necessarily related to symptoms development. This is probably related to the presence of latent infections.

The new IP-ELISA method is able to detect and localize bacterial antigens in plant tissue sections. The technique is very rapid (3 h), simple, useful for large scale analyses and cheaper than ELISA with extracts. The technique seems to be more sensitive than ELISA using MCA. Printed membranes can be developed immediately or several months later and can be readily mailed for developing. IP-ELISA opens new possibilities for diagnosis, detection and localization of bacterial antigens in field conditions, sanitation programmes and quarantine stations.

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Characterization of bacteria cross-reacting with antibodies against *Erwinia chrysanthemi*

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Abstract

Eighteen strains of bacteria, cross-reacting with antibodies against *Erwinia chrysanthemi* (Ech), were isolated from potato peel extracts, ditch water and the rhizosphere of wheat, onion, sugar beet and chicory. Sixteen strains were isolated through immunofluorescence colony-staining (IFC), while two strains were obtained by random isolation. For this purpose fluorescent colonies in IFC-preparations were punctured and isolated cells were subsequently suspended and dilution plated. Based on fatty acid profiles, these strains were all classified as fluorescent *Pseudomonas* species.

These bacteria cross-reacted with polyclonal antibodies against Ech in immunofluorescence cell-staining, Ouchterlony double diffusion and in ELISA. They also reacted in ELISA with monoclonal antibodies against the lipopolysaccharides of Ech were detected.

Cell envelope extracts and proteinase K treated cell envelope extracts (mainly lipopolysaccharides) of the cross-reacting strains were analysed with SDS-PAGE and Western blotting. Based on protein patterns and lipopolysaccharide patterns the cross-reacting bacteria could be classified in four groups. Indications were obtained that in all cases the lipopolysaccharide O-chains were responsible for the cross-reactions.

Additional keywords

ELISA, immunofluorescence cell-staining, immunofluorescence colony-staining, Ouchterlony double diffusion, SDS-PAGE, Western blotting, lipopolysaccharides

Introduction

Fluorescent *Pseudomonas* strains that cross-react with antibodies against *Erwinia chrysanthemi* (Ech) were isolated through immunofluorescence colony-staining (IFC) (VAN VUURDE & ROOZEN, 1990) from different substrates, during studies on sources reponsible for contamination of seed potatoes with Ech.

In this study the reaction levels of the cross-reacting strains in various serological assays were estimated. The antigenic components responsible for the cross-reactions were identified with SDS-PAGE and Western blotting.

Materials and Methods

Bacterial strains and growing conditions. Cross-reacting bacteria were characterized by the Plant Protection Service (J.D. Janse) with fatty acid profiling as fluorescent *Pseudomonas* isolates. Two strains, S44 and S45, were obtained by random screening of bacterial colonies from a potato peel extract (VAN DER WOLF & GUSSENHOVEN, 1992). The other 16 cross-reacting bacteria were isolated through IFC. Only data of a selection of 6 strains are given (Table 1). Ech IPO strain nr. 502 and *E. carotovora* subsp. *atroseptica* (Eca) IPO strain nr. 161 were used as positive and negative control, respectively. Bacterial suspensions were prepared from cultures grown for 24 h at 27 °C on trypticase soy agar (BBL) slopes.

Antiserum preparation. Polyclonal antisera were produced against total cell extracts or whole cells using the immunization protocol as described by VRUGGINK & MAAS GEESTERANUS (1975). Antiserum 9024C and 8276B were prepared against Ech 502, antiserum 9053C was prepared against Ech RH6050, a mutant of Ech 3937jRH which lacks the lipopolysaccharide (LPS) O-chain (SCHOONEJANS *et al.*, 1987). Monoclonal antibodies (mca) 2A4 were directed against the LPS O-chain. Immunoglobulin purification and conjugation of immunoglobulins with FITC or alkaline phosphatase was carried out as described by VAN DER WOLF & GUSSENHOVEN (1992).

ELISA. Threshold levels were determined in a double antibody sandwich ELISA using antiserum 8276B (VAN DER WOLF & GUSSENHOVEN, 1992). ELISA titers with mca 2A4 were determined in a triple antibody sandwich format as follows: Immunoglobulins from antiserum 8276B were used as coating antibodies (18 h, 4 °C). Coated plates were subsequently incubated with bacterial suspensions (10^8 cells/ml, 18 h, 4 °C), mca 2A4 (28 μg/ml, 2 h, 27°C) and goat-anti-mouse phosphatase (Dakopatts, D314) diluted 1/1000 (1 h, 27 °C).

Ouchterlony double diffusion (ODD). ODD was performed according to VAN DER WOLF & GUSSENHOVEN (1992) using antiserum 9024C.

Immunofluorescence cell-staining (IF). IF was carried out in an indirect way with goat-anti-rabbit FITC as described by VAN VUURDE *et al.* (1983).

Cell envelope preparation. Cell envelopes were prepared according to DE WEGER *et al.* (1987). Crude lipopolysaccharide extracts were prepared by a treatment of cell envelopes, dissolved in SDS sample buffer, with 5 $\mu g.ml^{-1}$ proteinase K for 30 min at 56 °C.

SDS-PAGE. SDS-PAGE was carried out as described by LAEMMLI (1970) using 15% (w/v) separation gels and 3% (w/v) stacking gels. Proteins and LPS were stained as described by FRANKEN *et al.* (1992).

Western blotting. Western blotting was carried out as described by FRANKEN *et al.* (1992) using rabbit antibodies in a dilution of 1/100 and goat-anti-rabbit phosphatase (Sigma, A9919) in a dilution of 1/10 000.

Results

Based on preliminary research with SDS-PAGE and Western blotting, 6 representative strains were selected from a collection of 18 cross-reacting *Pseudomonas* strains for further analyses. Reaction levels of these strains with Ech antibodies were compared with the reaction levels of Ech in ELISA (antiserum 8276B and mca 2A4), ODD and IF (Table 1). Apart from a low reaction level of S67 with mca 2A4 in ELISA, relatively strong cross-reactions were found in all assays.

Based on the patterns of the major cell envelope proteins in SDS-PAGE, the 18 strains can be divided in two main groups (A and B), which can both be subdivided in two subgroups (A1 and A2; B1 and B2). Representatives of these subgroups are shown in Fig. 1. Classification in subgroups is based on relative small differences in the patterns of the major cell envelope proteins.

Proteinase K treated cell envelopes were analysed with SDS-PAGE, too. Based on the thus obtained LPS-patterns, the 18 strains could be divided in 2 main groups (1 and 2); LPS group 1 and 2 correspond with cell envelope group A and B, respectively. Representatives of these groups are shown in Fig. 2.

An immunoblot of the cell envelope preparations probed with antiserum 9053C directed against a deep rough mutant of Ech is shown in Fig. 3. On this blot only a few (weakly reacting) protein bands of the cross-reacting

Pseudomonas strains are detected, whereas from Eca 161 and Ech 502 also a faint reaction with the LPS core part was found. In contrast, with antiserum 9024C, raised against a wild type strain of Ech, a strong reaction on blot was obtained with proteinase K treated cell envelopes, resulting in a dark smear along the lanes (Fig. 4).

Table 1. Reactions of saprophytes with antibodies against Ech in resp. ELISA, Ouchterlony double diffusion (ODD) and immunofluorescence cell-staining (IF).

Strain	Source[1]	ELISA threshold[2] (in cells/ml) antiserum 8276B	ELISA titre[3] (mca 2A4)	ODD titre	IF titre[4]
S44	potato	10E5[5]	ND	64	800
S65	onion	10E6	64000	64	1600
S67	wheat	10E7	1000	32	800
S70	wheat	10E6	16000	64	800
S71	wheat	10E5	32000	64	100
S92	ditch water	10E5	64000	64	1600
Ech 502	potato	10E6	>256000	128	1600
Eca 161	potato	nc	nc	<4	<25

1. Source from which the saprophytes were isolated. Potato = potato peel extracts. For the isolation of saprophytes from onion, wheat and sugar beet, rhizosphere extracts were used
2. Tested in a double antibody sandwich ELISA.
3. Serial dilutions of purified monoclonal antibodies were tested in a triple antibody sandwich ELISA.
4. Tested with an indirect IF
5. In the two ELISA experiments a reaction was considered to be positive when the avarage absorbance value exceeded the avarage absorbance value of the negative control (Eca, 10^9 cells/ml) supplemented with 3 times the standard deviation

ND = not determined, nc = negative control

Discussion

Based on the cell envelope protein patterns (Fig. 1) and the LPS-patterns (Fig. 2) the cross-reacting *Pseudomonas* strains could be divided into two main groups.

The high reaction levels of the cross-reacting *Pseudomonas* strains with the Ech antibodies indicate that a dominant antigen is involved in the cross-reactions. Strong indications were obtained that the immunodominant LPS O-chain is responsible for the cross-reactions, because:

1. Seventeen out of 18 strains cross-reacted strongly with mca 2A4 directed against the LPS O-chain of Ech.
2. Cell envelopes of the *Pseudomonas* strains treated with proteinase K cross-reacted strongly on blot with Ech antibodies (Fig. 4).

3. Only weakly reacting proteins of the *Pseudomonas* strains were detected when an antiserum raised against a rough mutant of Ech was used. Knowledge about components, responsible for cross-reactions will be helpful in producing more specific antibodies against Ech.

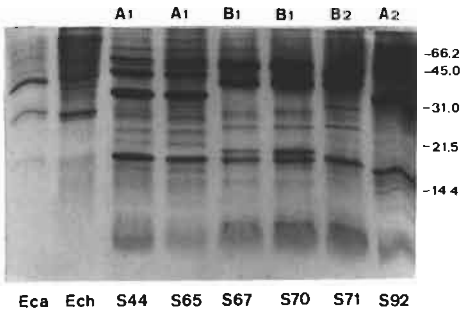

Fig. 1. SDS-PAGE patterns of cell envelopes of *Erwinia* and *Pseudomonas* strains, cross-reacting with Ech antisera. Above the lanes the cell envelope groups are indicated

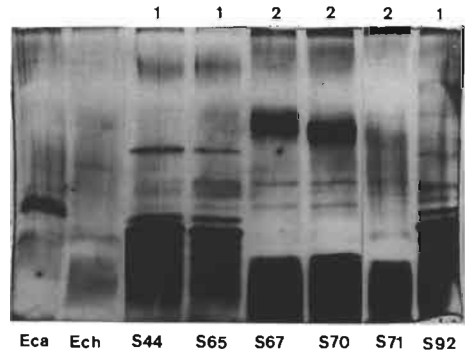

Fig. 2. SDS-PAGE of proteinase K treated cell envelopes of *Erwinia* and *Pseudomonas* strains, cross-reacting with Ech antisera. Above the lanes LPS groups are indicated.

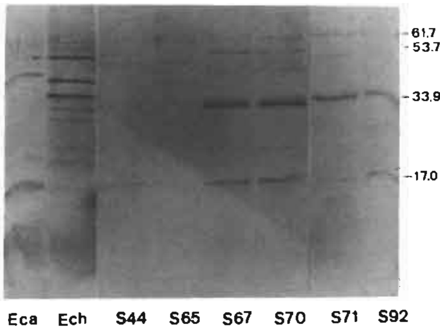

Fig. 3. Detection of cell envelopes on blot, using antibodies raised against a LPS O-chain lacking mutant

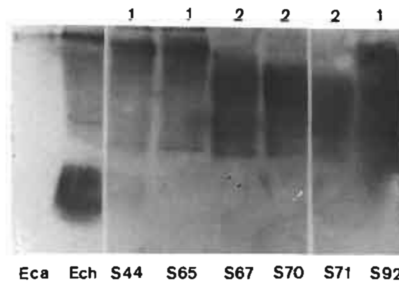

Fig. 4. Detection of proteinase K treated cell envelopes on blot, using antibodies against a wild type strain of Ech

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Contribution of isozyme analysis to the identification of *Pseudomonas syringae* pathovar *pisi*

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Distinction between *Pseudomonas syringae* pv. *pisi*, responsible of bacterial blight of pea (*Pisum sativum*), and the other pathovars of the same species, especially the pathovar *syringae*, still partly required strain inoculation onto peas. Enzymatic patterns study (esterases and superoxide dismutase) as a new identification way for this pathogen was realized. Profiles obtained for 59 *P.s.* pv. *pisi* strains, 53 *P.s.* pv. *syringae* strains and 2 strains of 11 other pathovars of *P. syringae* were studied. Pathovar *pisi* was characterized by a strong profile homogeneity for the tested strains (one unique profile for the SOD, two types of profiles for esterases), unlike pathovar *syringae* whose profiles were heterogeneous. SOD patterns did not allow the identification of *P.s.* pv. *pisi* strains (26.4% of *P.s.* pv. *syringae* strains and the *P.s.* pv. *aptata* strains showed a profile similar to that of *P.s.* pv. *pisi*). On the other hand, the two profiles obtained for the esterases were specific to the pathovar *pisi* strains. This method is proposed as an identification test for *P.s.* pv. *pisi*.

Key words : *Pseudomonas syringae* pv. *pisi*, identification, electrophoresis, isozyme.

INTRODUCTION

Pseudomonas syringae pv. *pisi* (*P.s.* pv. *pisi*) is the causal agent of bacterial blight of pea (*Pisum sativum*) first described by Sacket in 1916. This pathogen is

one of the about fifty pathovars of the species *Pseudomonas syringae* (DYE *et al.*, 1980 ; YOUNG *et al.*, 1991), species subdivision based on pathogenicity to different host plants. A problem of identification of *P.s.* pv. *pisi* is due to the epiphytic presence of *Pseudomonas syringae* pv. *syringae* (*P.s.* pv. *syringae*) on pea. Indeed since physiological criteria did not allow the distinction between these two pathovars, serological methods were tested (Taylor, 1972) as well as determination of O-serogroup (Saunier & Samson, 1987) combined with some physiological characters (hydrolysis of esculin and production of fluorescent pigment) (Grondeau *et al.*, 1991), but neither led to a 100% safe diagnosis.
The isozyme analysis as a possible new way for *P.s.* pv. *pisi* identification was evaluated in this study.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A sample of 59 strains of *P.s.* pv. *pisi* from various geographical origins, O-serogroups and races, was tested in comparison to 53 *P.s.* pv. *syringae* strains isolated in FRA from pea (except the type-strain of the pathovar CFBP 1392^T isolated in GBR from lilac). Two strains of different other pathovars (*aptata*, *glycinae*, *helianthi*, *lachrymans*, *mors-prunorum*, *persicae*, *phaseolicola*, *porri*, *savastanoï*, *tabaci* and *tomato*) were added to the study. Bacteria were grown 48 h on King B plates at 23°C.
Preparation of extracts and electrophoresis. Cells from two plates were suspended in extraction buffer (Some & Samson, 1991) and sonicated in an ice bath. After 2 centrifugations, supernatant was frozen at -20°C until use. Proteins were separated using a vertical polyacrylamide gel (11% acrylamide) with a constant voltage of 60 volts/gel for 2 h at 4°C. Two *P.s.* pv. *pisi* strains were loaded on each gel as references.
Staining procedures. Isozyme polymorphism was investigated at two enzymatic loci : esterases (EST) and superoxide dismutase (SOD) with the staining procedure described by Siciliano & Shaw (1976). The electrophoretic mobility of a given variant was measured as the relative mobility value (MR) which is the ratio between the distance measured for the band and the total migration distance.

RESULTS - DISCUSSION

***P.s.* pv. *pisi* patterns**. The 59 *P.s.* pv . *pisi* strains showed for SOD a single profile of five enzymatic bands with MR values 0.25 ; 0.27 ; 0.62 ; 0.67 and 0.87. For EST, the strains were divided into two zymotypes. The first zymotype, representing 78% of the tested strains, was composed of six bands (MR values : 0.47 ; 0.58 ; 0.62 ; 0.69 ; 0.71 and 0.78). The second zymotype with five bands (22% of the tested strains) differed from the first one in the MR value of the first band (0.49), the absence of the fourth band and a more intense fifth band. *P.s.* pv. *pisi* was characterized on the basis of these results by a high homogeneity without any influence of geographical origin of the strains. The existence of two zymotypes within EST profiles seemed correlated with the race system (TAYLOR *et al.*, 1989). All the strains belonging to the zymotype 2 were indeed determined by J. Taylor as races 1 or 5, and no strains of the zymotype 1 belonged to races 1 or 5.
***P.s.* pv. *syringae* patterns**. Unlike *P.s.* pv. *pisi*, *P.s.* pv. *syringae* strains presented a high heterogeneity in profiles for the two enzymatic loci examined . The 53 strains tested were divided into 9 SOD zymotypes. The main zymotype (26.4% of the tested strains) had a pattern identical to that obtained for *P.s.* pv. *pisi* strains. The other *P.s.* pv. *syringae* strains were distributed mostly among 4 other zymotypes, each concerning respectively 18.8%, 17%, 15% and 9.4% of the strains and different from *P.s.* pv. *pisi* pattern by one or two bands. This heterogeneity was still more marked with the EST profiles : 40 different patterns for 53 strains. Among these 40 patterns nonewas identical to zymotype 1 or 2 of *P.s.* pv. *pisi* strains.
The other pathovars of *P. syringae*. The two strains of each of the 11 other pathovars tested presented in most cases the same pattern, except the 2 strains of the pathovar *mors-prunorum* which exhibited 2 very dissimilar patterns for SOD and EST. EST patterns for the two strains of pathovars *tomato* and *persicae* were only slightly different. For SOD, some pathovars presented the *P.s.* pv. *pisi* pattern (*aptata*) or one of the *P.s.* pv. *syringae* patterns (*helianthi*, *persicae*, *tomato* and *porri*). The others had distinguishing profiles. The EST patterns of the 11 tested pathovars were different from those of *P.s. pv.* pisi.

SOD profiles are of no help for identification of *P.s.* pv. *pisi* since 26.4% of *P.s.* pv. *syringae* strains and the pathovar *aptata* strains exhibit the same profile.

But EST patterns obtained for *P.s.* pv. *pisi* seem to be highly specific of this pathovar and can be used as a complementary identification test for *P.s.* pv. *pisi.*

RESUME

La distinction entre *Pseudomonas syringae* pv. *pisi* responsable de la graisse du pois (*Pisum sativum*) et les autres pathovars de la même espèce, particulièrement le pathovar *syringae*, nécessite encore en partie l'inoculation sur le pois. L'étude des profils enzymatiques (estérases et superoxyde dismutase) en tant que nouvelle voie d'identification a été réalisée. Les profils obtenus pour 59 souches de *P.s.* pv. *pisi*, 53 souches de *P.s.* pv. *syringae* et 2 souches de 11 autres pathovars de *Pseudomonas syringae* ont été étudiés. Le pathovar *pisi* est caractérisé par une très grande homogénéité de profil pour les souches testées (un profil unique pour la SOD, deux types de profil pour les estérases), contrairement au pathovar *syringae* dont les profils sont hétérogènes. Les profils SOD n'ont pas permis l'identification des souches de *P.s.* pv. *pisi* (26,4% des souches de *P.s.* pv. *syringae,* et les souches du pathovar *aptata* présentent un profil similaire à celui de *P.s.* pv. *pisi*). Par contre, les deux profils obtenus pour les estérases sont spécifiques des souches du pathovar *pisi.* Cette méthode peut être utilisée comme méthode d'identification de *P.s.* pv. *pisi.*

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The use of conductimetric assays for the detection of *Pseudomonas syringae* pv. *pisi* in pea seeds

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Introduction

Test protocols for detecting *Pseudomonas syringae* pv. *pisi* (Pspi), the causal agent of bacterial blight, in pea seeds are generally based on dilution-plating assays of pea seed extracts. These assays are usually very specific and reliable. However, for rapid large scale screening of seed lots less laborious and time-consuming methods are needed. The use of conductimetric assays have shown their potential for rapid screening of food products for food-borne bacteria (EDEN & EDEN, 1984; BOLTON, 1990).

The aim of this study was to investigate the potential of conductance methods to index seed lots for presence of Pspi. Different extraction procedures, media and incubation temperatures were examined for direct conductance measurement experiments.

Materials and Methods

Thousand seeds per seed lot were either 6 h soaked, or ground and 2 h soaked in sterile water. Sterile water was added to the non-ground samples in a quantity corresponding to 2.0 ml multiplied by the weight (in grams) of 1000 seeds. A double amount of water was added to the ground samples. After soaking, seed extracts were spiked with cell suspensions of Pspi strain 518. Cell concentrations were determined on King's medium B (KB, KING *et al.*, 1954) and 5 % sucrose nutrient agar (SNA, TAYLOR & DYE, 1972), containing boric acid, cephalexin and cycloheximide (SNAC) as reported by MOHAN & SCHAAD (1987). Conductance

responses at 17 and 27 °C were measured with a Malthus 2000 analyser using 8 ml cells to which 2 ml of SPYEC (Special Peptone Yeast Extract containing boric acid, cephalexin, cycloheximide and cefuroxime) was added. One ml of bacterial suspension or seed extract was added to each cell. The default scan rate used was 18 minutes and detection time (time at which the first significant conductance change appears), maximum rate of conductance change (μS/h) and the maximum conductance change (μS) were determined. After detection in conductimetry, samples were taken and checked for presence of Pspi with *Staphylococcus aureus*-agglutination (LYONS & TAYLOR, 1990) and immunofluorescence microscopy (IF, VAN VUURDE *et al.*, 1983), using polyclonal antibodies. These samples, 1:10000 diluted, were also plated on KB and SNAC in order to isolate and identify Pspi colonies.

Results

Detection times (Td) obtained in conductimetry were correlated with cell concentrations of Pspi in seed extracts. Fig. 1 shows there is a linear relationship between Td and cells added to the extracts. However, the slope of these lines is depending on the extraction method used. Detection of Pspi 518 in the ground and 2 h soaked pea seed samples took longer than the detection of pure cultures, while the opposite was found for the 6 h soaked pea seed samples. This can be explained by the release of toxic substances from the flour of the ground seeds. The shorter Td for the 6 h soaked samples, as compared to the pure cultures, can be explained by leakage of Pspi growth stimulating substances from the seeds. Typical conductance curves for both extraction methods are given in Fig. 2A and 2B.

In spite of the use of a semi-selective medium some interference of saprophytes may still occur. This interference can usually be deduced easily from the shape of the conductance as can be seen in Fig. 3A/B and 4. The interference of saprophytes is less at lower temperature (Fig. 3B). However, the detection times are much higher through a slower growth of Pspi. The conductance responses are also decreasing with lower temperatures (Fig. 3B).

For seed lots, yielding detection times shorter than those corresponding to

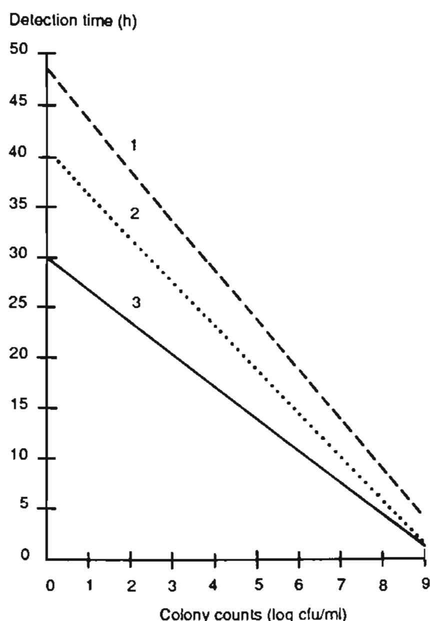

Fig. 1. The relation between detection times in conductimetry and Pspi colonies added to the seed extracts. Conductimetric assays were done in SPYEC at 27 °C.
1= detection of Pspi 518 in ground and 2 h soaked pea seed samples,
Y = -4.97X + 48.75
2= detection of Pspi 518 as pure culture,
Y = -4.33 + 40.58
3= detection of Pspi 518 in 6 h soaked pea seed samples,
Y = -3.18X + 29.94
The coefficients of correlation were -0.94 (n = 39), -0.94 (n = 15) and -0.93 (n = 31) for lines 1, 2 and 3, respectively.

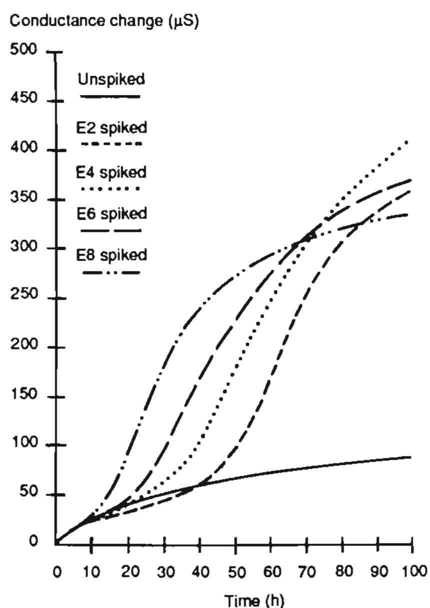

Fig. 2A. Conductance responses in SPYEC at 27 °C as a result of growth of Pspi 518 in extracts of ground and 2 h soaked pea seeds of seed lot A. Ex = 10^x cfu per ml at the start of the test. No growth of bacteria in the unspiked sample

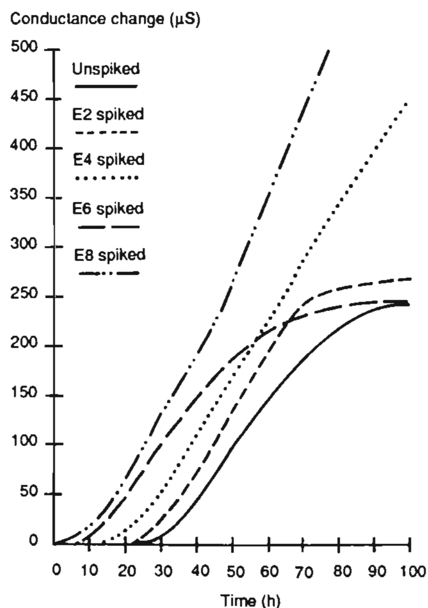

Fig. 2B. Conductance responses in SPYEC at 27 °C as a result of growth of Pspi 518 in extracts of the 6 h soaked pea seeds of seed lot A. Only Pspi was isolated from the unspiked sample.

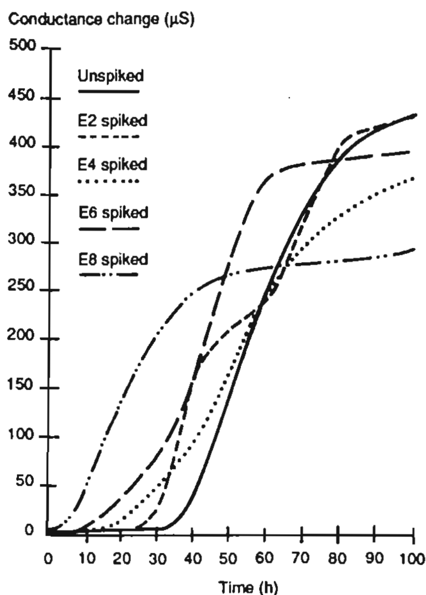

Fig. 3A. Conductance responses in SPYEC at 27 °C, initially as a result of growth of Pspi 518 in the 6 h soaked pea seed samples of seed lot B. Except for the 10^8 cfu/ml-spiked sample, growth of saprophytes was found after 30 h for all samples.

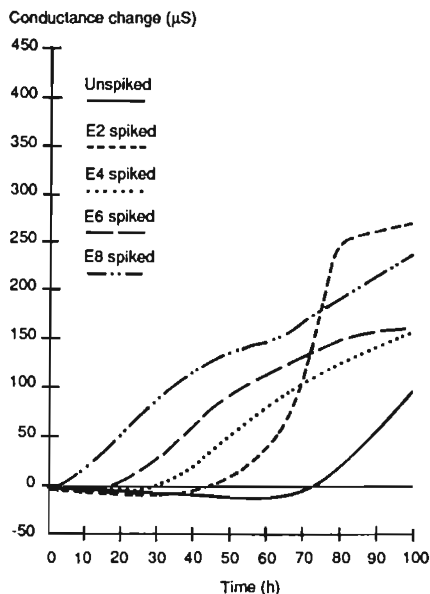

Fig. 3B. Conductance responses in SPYEC at 17 °C, initially as a result of growth of Pspi 518 in the 6 h soaked pea seed samples of seed lot B. After 60 h, only growth of saprophytes in the 10^2 cfu/ml-spiked and unspiked samples.

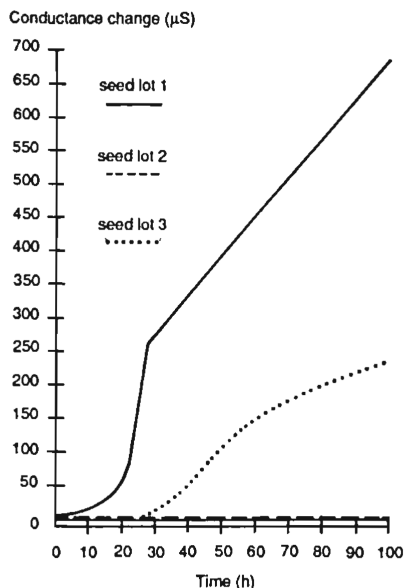

Fig. 4. Conductance responses at 27 °C of the microflora from several seed lots in SPYEC.
Note: Mainly growth of a levan-producing, oxidase positive, green fluorescent pseudomonad in the 6 h soaked pea seed samples of seed lot 1, no growth in the samples of seed lot 2 and growth of Pspi in the samples of seed lot 3.
Seed lot 3 was also found positive with dilution-plating on SNAC.

log cfu/ml = 0, as determined by the calibration lines (Fig. 1), it is necessary to check and confirm the conductance results by application of dilution-plating and rapid serological tests, such as IF and *Staphylococcus aureus*-agglutination. Samples of conductivity cells were taken after c. 20, 40 and 60 h of incubation in SPYEC at 27 °C. Fig. 4 shows the conductance responses of 3 recently tested pea seed lots. Only the 40 and 60 h samples of seed lot 3 were found positive with *Staphylococcus aureus*-agglutination and IF. Long strands of dividing fluorescent cells were typical for the 40 h sample, whereas the 60 h sample yielded mainly weakly stained cell debris with a few whole cells. The 'negative' samples of detected conductivity cells, tested so far, yielded mainly oxidase positive, fluorescent, levan- or no levan-producing *Pseudomonas* strains.

Discussion and conclusions

The sensitivity and specificity of the conductimetric assay is sometimes depending on the number of saprophytes, mainly *Pseudomonas spp.*, which can overgrow Pspi and cause conductance change responses in the media used. More (naturally contaminated) seed lots are now being tested to precise and establish the exact confidence intervals of the calibration lines, as shown in Fig. 1. The Td obtained in conductimetry can be used to decide whether a seed lot should be regarded as 'healthy' (e.g. seed lots with Td above 30 h when using 6 h soaking of seed lots) or potentially infested by Pspi (e.g. seed lots with Td below 30 h when using 6 h soaking of seed lots). Presence of Pspi in suspected seed lots can be confirmed by other rapid techniques, such as serological techniques. With regard to speed, sensitivity and simplicity, the use of conductimetric assays for the detection of Pspi in seed extracts has a high potential for application on a large scale. Further research will focus on improvement of media-selectivity in order to avoid growth of saprophytes, as mentioned before.

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Pathogenicity and variability of *Xanthomonas campestris* from avocado canker in California

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Canker symptoms have been observed on trunks and branches of avocado in South Africa and California. In South Africa, the cankers are associated with *Pseudomonas syringae*, but similar symptoms in California are associated with *Xanthomonas campestris*. *X. campestris* was isolated from 14 avocado groves with canker symptoms in five counties of California. No *P. syringae* was recovered from any of the samples. Isozyme analyses, restriction fragment length polymorphism (RFLP) analyses, DNA fingerprinting with rare-cutting enzymes, Biolog carbon source utilization tests, and pathogenicity assays were employed to compare 26 isolates of the California avocado pathogen. There was considerable genetic variation between isolates and variation in the aggressiveness toward avocado seedlings, as measured by distance of systemic movement and necrosis. The avocado isolates were related to *X. campestris* but distinct from other pathovars that were compared.

INTRODUCTION

Bacterial canker of avocado was described in South Africa in 1982 (Myburgh & Kotze, 1982), and similar symptoms have been observed on trunks and branches of avocado trees in southern California groves for many years. Lesions appear as slightly sunken and darker areas on the bark, with a necrotic, watery pocket under the sunken area. As cankers develop, the bark splits, and fluid oozes out and dries to a powdery-white residue. Necrotic streaks usually extend from the necrotic area

below the cankers and often connect two or more cankers along a branch or trunk. *Pseudomonas syringae* was identified as the causal agent of bacterial canker of avocado in South Africa, but in California, *Xanthomonas campestris* is associated with these symptoms and has been shown to be pathogenic on avocado (Cooksey *et al.*, submitted).

X. campestris was isolated from from 26 avocado samples with bacterial canker symptoms from 14 groves in five counties of California. Inoculation of five of these strains to Hass avocado plants resulted in a spreading vascular and pith necrosis accompanied by spread of the bacterium in these tissues. The bacterium and necrotic symptoms never spread below the graft union of inoculated plants, however. No disease symptoms were produced after inoculation of the bacterium to avocado leaves (Cooksey *et al.*, submitted).

In this study, 26 strains of *X. campestris* from avocado canker symptoms in California were tested for variation in pathogenicity and for genetic variation by several methods. Their relatedness to other *X. campestris* pathovars was also investigated.

MATERIALS AND METHODS

Pathogenicity. Each bacterial strain was inoculated to two Hass seedlings by making a 5-mm-long wound with a razor blade about 15-20 cm above the graft union and placing 20 μl of a bacterial suspension (1.5×10^{11} cfu/ml) onto the wound. After 10-11 weeks, stems of each seedling were surface sterilized and cut into 1-cm sections. The sections were weighed and diced in a known volume of sterile water for dilution-plating onto a semiselective tween agar medium (McGuire *et al.*, 1986).

Analysis of genetic variation. Isozyme analysis was performed by horizontal starch gel electrophoresis with 15 enzyme assays. For RFLP analysis, Southern blots of total DNA digested with *Eco*RI were probed with cosmid clones containing Hrp (hypersensitive reaction and pathogenicity) genes (Lindgren *et al.*, 1987) from either *X. campestris* pv. vesicatoria or *P. syringae* pv. tomato with stringent wash conditions. Genomic fingerprinting was performed by pulsed-field gel electrophoresis of total DNA digested with a rare-cutting enzyme, *Xba*I (Cooksey &

Graham, 1989; Egel *et al.*, 1991). Biolog carbon source utilization tests were performed as described by the manufacturer. Similarity coefficients were calculated from isozyme, RFLP, genomic fingerprinting, and Biolog data, and cluster analysis was performed with the NTSYS-pc program.

RESULTS

Pathogenic variation. Of 26 strains of *X. campestris* isolated from avocado cankers, 20 strains spread from the inoculation point and caused an internal necrosis in avocado stems. The other six strains were apparently nonpathogenic. The extent of spread within stems varied among strains, suggesting variation in pathogenicity. While most strains did not spread or cause necrosis below the graft union, two strains did spread well below the graft union and also caused necrosis well into the rootstock. *X. campestris* pv. campestris was nonpathogenic; it did not spread or cause necrosis beyond the inoculation point.

Genetic variation. Isozyme analysis indicated variability among the 26 avocado strains of *X. campestris*, but all isolates except one were related, with similarity coefficients greater than 0.72. Fifteen isolates, all from Ventura and Orange counties, formed a cluster with similarity coefficients greater than 0.88. A separate cluster showed strong similarities between two strains from San Diego county and one from Ventura county. A single isolate from Santa Barbara county was not closely related to *X. campestris* from avocado or the other *X. campestris* pathovars tested, and its putative identification as *X. campestris* needs to be reexamined. A strain of *X. campestris* pv. campestris used as a reference showed coefficients of similarity with the avocado isolates of 0.71-0.77. *X. campestris* pv. vesicatoria was more closely-related to the avocado strains and clustered with two avocado strains of *X. campestris* from Los Angeles county. Surprisingly, *P. syringae* from South African avocado canker (Myburgh & Kotze, 1982), originally included as an unrelated reference strain, showed strong similarity with *X. campestris* pv. vesicatoria (0.81) and the avocado strains of *X. campestris* from Los Angeles county (0.77-0.84).

A cosmid clone containing Hrp genes from *X. campestris* pv. vesicatoria hybridized with 4-7 *Eco*RI fragments from the genome of the avocado isolates of *X.*

campestris, except for the one strain from Santa Barbara county that showed low similarity with *X. campestris* by isozyme analysis. The relatedness of a large cluster of 15 strains from Ventura and Orange counties, identified previously by isozyme anlysis, was confirmed by RFLPs using the Hrp probe. In addition, the two strains from San Diego county and one from Ventura county that clustered together by isozyme analysis were identical by RFLP analysis. Hybridization was also observed between this Hrp gene probe and *P. syringae* from avocado in South Africa and with *P. syringae* pv. tomato. However, a cosmid clone containing Hrp genes from *P. syringae* pv. tomato hybridized strongly to *P. syringae* from avocado in South Africa but not to *X. campestris* from avocado in California or other reference strains of *X. campestris*.

Genomic fingerprinting by *Xba*I digestions of total DNA revealed several subgroups of 2-4 strains with identical banding patterns within the subgroups. This analysis confirmed the close relatedness between most of the Ventura and Orange county strains, as suggested by isozyme analysis. The similarity of two strains from San Diego county and one from Ventura county suggested by isozyme and RFLP analyses was also confirmed by genomic fingerprinting.

Biolog analysis confirmed that the avocado isolates of *X. campestris* were related. Again, a large cluster of related strains mostly from Ventura and Orange counties was identified, as well as a second cluster of two San Diego county and one Ventura county strains that was also identified by isozyme, RFLP, and fingerprinting methods. This latter group of three strains was the most closely-related of the avocado strains to reference strains of *X. campestris* pv. campestris, *X. campestris* pv. vesicatoria, and *X. campestris* pv. translucens. A search of the Biolog GN database (Version 3.0) indentified 11 pathovars of *X. campestris* as the closest matches to these three strains.

DISCUSSION

Strains of *X. campestris* from avocado canker in California show considerable genetic and pathogenic variability but can be considered as a related group. They are apparently distinct from other *X. campestris* pathovars but are generally related to the *X. campestris* group. Together with the observations of canker symptoms in

California avocado groves for many years, the variability among strains of this pathogen suggest that it has been present for a considerable time in this area and does not indicate the recent introduction of a homogeneous group. However, strong similarities between some strains from different counties in California may indicate that some movement of the bacteria between these areas has occurred, or that some groves have received similar strains from common sources of stock plants.

There was considerable variation in pathogenicity, as determined by the extent of spread of the bacterium and necrotic symptoms in avocado stems over a 10-11 week period. Some strains appeared to be nonpathogenic, although genetic analysis suggests that they are highly related to pathogenic strains from similar areas. Two strains were able to spread downward in avocado stems past the graft union. This had not been observed previously after inoculation of other strains or by observation of field and nursery samples that had natural infections. These two strains may represent more aggressive forms of the pathogen, but they were not closely related by genetic analysis. *X. campestris* pv. campestris caused no necrosis and did not spread in avocado stems, but this was the only other pathovar that we tested for pathogenicity on avocado. Whether this disease is caused by a single pathovar uniquely adapted to avocado or by a mixture of strains from other hosts (Graham *et al.*, 1990) has not yet been determined.

ACKNOWLEDGEMENT

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Polymerase chain reaction used in the development of a DNA probe to identify *Erwinia stewartii* a bacterial pathogen of maize

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ABSTRACT

Erwinia stewartii (Smith) Dye causes stewart's wilt, a bacterial disease of maize responsible for serious crop losses and for which there is evidence of seed transmission. At present, seed health tests are time consuming and lack sensitivity. This project aims to develop quicker and more effective methods for detecting *E.stewartii* from other *Erwinia* and *Pseudomonas* species. A molecular probe is being used to screen more isolates of *Erwinia* species before cloning and sequencing and further development into a new seed health test.

KEYWORDS

Erwinia stewartii, seed health test, quarantine, RAPD, PCR, DNA probe, maize pathogen.

INTRODUCTION

Erwinia stewartii (Smith) Dye causes Stewart's wilt disease in maize, which is responsible for serious crop losses in many countries. There is evidence that this disease can be seed transmitted as well being spread by the corn flea beetle *Chaetocneme pulicaria.* The thorough exploitation of maize genetic resources is constrained by seedborne disease organisms which could either cause the seed to fail to meet import legislation standards or introduce disease to other regions. It is therefore essential that germplasm collections are free of such pathogens. This can be determined by appropriate seed health testing. Detection and identification techniques using PCR have been used for other organisms but DNA sequence information is required for the synthesis of specific primers for each organism and obtaining these data can be laborious. Recent work on PCR, using arbitrary primers requiring no prior sequence information (random amplified polymorphic DNA-RAPD), has revealed DNA polymorphisms that may be useful for fingerprinting genomes (Reeves and Ball, 1991; Welsh and McClelland, 1990; Williams, Kubelik, Livak, Rafalkski and Tingey, 1990).

METHODS

Twenty three *E.stewartii* isolates, eleven *Erwinia chrysanthemi* isolates, twelve *Erwinia salicis* isolates, nine *Erwinia herbicola* isolates, four *Erwinia carotovora* isolates, two *Erwinia rhapontici* and two *Pseudomonas syringae pv. pisi* isolates were used. Except for strains of E.salicis, a single colony from each bacterial isolate was inoculated into 100ml of nutrient broth and incubated at 25°C on an orbital shaker until mid-log phase. Strains of *E.salicis* were inoculated and incubated as above but using nutrient broth with 1% glycerol. The bacterial cells were then harvested by centrifugation and DNA was extracted by the procedure of Silhavy, Berman and Enquist, (1984). PCR was performed in 50ul volumes using a ten base primer (primer 70) and under amplification conditions as given in Reeves and Ball, (1991). The PCR products were then fractionated on a 1% agarose gel and stained with ethidium bromide. Genomic DNA from a variety of bacterial isolates including E.stewartii was dot blotted onto a nylon membrane. The 1.58kb DNA band amplified in *E.stewartii* strain 3253 was excised from an agarose gel and radioactively labelled with αP^{32}dCTP. The dot blot membrane was probed using this labelled fragment. Hybridisation was carried out for 24 hours at 65°C under high stringency conditions (Maniatis, Fritsch and Sambrook, 1982) and detected by autoradiography.

RESULTS

DNA from seven isolates of *E.stewartii,* amplified using primer 70, shared an intense 1.58Kb band which was not observed in other *Erwinia* or *Pseudomonas* species . When the annealing temperature was raised by 5°C to 40°C in order to increase primer specificity, some of the less obvious amplified bands that were observed at the lower annealing temperature were not present (data not shown). However, the intense 1.58Kb band was observed at both temperatures indicating a higher specificity for primer 70. Only the DNA from the *E.stewartii* isolates hybridised with the *E.stewartii* 1.58Kb DNA probe on the dot blot membrane .

Further screening of other *E.stewartii* isolates and other bacteria likely to be within or on maize seed will be necessary to determine the specificity of the DNA probe. This probe will be sequenced to provide primers for specific PCR, the products from which can be detected using the probe with a non-radioactive label. From the

results of this research a new, rapid and sensitive seed test could be developed for use world wide.

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Distinction between tumorigenic and nontumorigenic agrobacteria using a T-DNA probe in nonradioactive systems

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Abstract

A study was carried out to verify the potential of using a T-DNA probe, pTHE17 in nonradioactive detection systems for the differentiation of tumorigenic and nontumorigenic *Agrobacterium* strains obtained from different origins. Hybridization tests were performed after different labeling of the probe: (a) random primed DNA with digoxigenin d-UTP and (b) nick translation using biotin 14d-ATP or 7d-ATP. The DNAs of *Agrobacterium* strains were extracted using alkaline lysis, denatured and blotted on membranes. The detection was done according to the "Genius nonradioactive nucleic acid detection system" (Boehringer Mannheim), using both color and light (Lumi-Phos 530) reaction, and according to "PhotoGene" and "BluGene" nucleic acid detection systems respectively (Bethesda Research Laboratory). With a few exceptions the DNA extracted from tumorigenic strains gave positive hybridization, indicating a close relationship between signal and tumorigenicity. However, hybridization experiments revealed some reactions to the DNA of nontumorigenic strains under standard conditions of the system used.

Key words: tumorigenic, nontumorigenic agrobacteria; DNA probe; identification; nonradioactive systems.

Introduction

Crown gall *(Agrobacterium tumefaciens,* AT) is a worldwide occurring bacterial disease which can affect economically important crops such as stone and pome fruit trees, grapevines and ornamentals (Kerr, 1992). Survival and spread of this pathogen in asymptomatic propagation material (latent infections) is great. In certification programs precautions must be taken to guarantee the phytosanitary state of mother plants.

Research has been initiated in several countries to improve reliable indexing methods for a large scale selection of propagation material free from endophytic tumorigenic agrobacteria. Different indexing methods have been proposed: some are based on serological procedures and/or protein gel electrophoresis (Bazzi *et al.*, 1987; Alarçon *et al.*, 1987; Burr *et al.*, 1988; Bishop *et al.*, 1989); whereas others are based on direct isolation of bacteria and their identification using total cellular fatty acid analysis (Jäger *et al.*, 1989). Burr *et al.* (1990) have shown the possibility to use 32P-labeled DNA probes for determining tumorigenicity of *Agrobacterium* strains. To overcome the problems related to the use of radioisotopes it seemed useful to verify the potential of using a T-DNA probe for identification and diagnosis of AT in nonradioactive detection systems.

Table 1. Sources of *Agrobacterium* strains and results of dot blot hybridization detection methods.

Strains	Biovar	Tumor.	Source		Lumi-Phos	PhotoGene	Genius	BluGene
1. CG 111	3	t	Grape gall	USA	++	+++	++	++
2. CG 112	3	t	Grape gall	"	++	++	++	++
3. CG 113	3	t	Grape gall	"	+	+	-	+
4. CG 56	3	t	Grape gall	"	+	++	++	++
5. CG 109	3	t	Grape gall	"	++	+++	++	+
6. CG 49	3	t	Grape gall	"	+	+++	++	++
7. CG 1023	1	t	Raspberry gall	"	++	+	++	+
8. CG 1024	1	t	Pear roots	"	++	++	++	+
9. CG 1035	nd	nt	Broccoli gall	"	-	++	+/-	+
10. CG 1032	nd	nt	Broccoli gall	"	-	-	-	++
11. CG 234	3	nt	Grape sap	"	-	+++	-	+++
12. CG 1027	3	nt	Tissue culture grape gall	"	-	+	-	+
13. CG 1028	1	t	Cherry gall	"	++	++	++	+
14. CG 1030	2	t	Cherry gall	"	+	+	+	+
15. CG 1019	1	t	Raspberry gall	"	++	+++	++	-
16. CG 1018	1	t	Raspberry gall	"	+++	+++	+++	+
17. CG 1029	2	t	Cherry gall	"	+++	+	++	+
18. CG 1031	2	t	Cherry gall	"	+++	++	++	+
19. CG 1022	1	t	Raspberry gall	"	++	+++	++	+
20. IPV-BO 2442	3	nt	Grape/graft gall	Italy	-	+	-	+++
21. K 84	2	nt	Soil	Australia	++	+	+/-	+
22. NCPPB 1651	2	t	Peach gall	South Africa	+++	+++	++	+
23. IPV-BO 1506	2	t	Rose gall	Italy	+++	+++	+++	+
24. CG 429	1	nt	Grape soil	USA	-	+++	+	+
25. IPV-BO 2150a	1	nt	Grape gall	Italy	-	-	-	-
26. CG 102	3	t	Grape gall	USA	+	++	++	+++
27. CG 210	1	nt	Grape soil	"	-	-	+	+
28. DH-1 (*E. coli*) pTHE17				USA	+++	+++	+++	+
29. 15834 (*A. rhizogenes*)				Italy	nd	++	nd	-
30. 360-1	1	nt	Grape gall	Spain	-	-	+/-	+
31. IPV-B0 2028	1	t	Daisy gall	Italy	++	+	++	+
32. HLB-2	1	nt	Hop gall	China	++	+++	+	+
33. IPV-B0 2147	3	t	Grape gall	Italy	++	+++	++	+
34. NCPPB 396	1	t	Dahlia gall	USA	+++	++	++	-
35. IPV-BO 1344	intermediate	t	Chrysanthemum gall	Italy	++	+	++	+

nd: not determined; t: tumorigenic; nt: nontumorigenic.
Index for signal intensity: +/-, very faint; +, faint; ++, medium; +++, dark; -, negative.

Materials and methods

DNA probe

The probe pTHE 17 of about 55 kilobases (supplied by G.T. Hayman and S.K. Farrand, University of Illinois, USA), cloned into *Escherichia coli strain* DH-1, contained all of the T-DNA from nopaline pTi of strain C58 (AT biovar 1) except for the far left portion. The probe included EcoRI fragments 16, 32, 28, 14, 4 omega and one with fragment numbering according to Depicker *et al.* (1980).

DNAs extracts

24 tumorigenic strains (including 3 biovars of AT and *A. rhizogenes)* and 10 nontumorigenic (Table 1) were grown on 523 agar medium (Kado *et al.*, 1972) at 27°C for 72 hours; E. *coli* strain DH-1 harboring pTHE17 recombinant plasmid was used as a control. Nucleic acid extraction and preparation of membranes were carried out according to the alkaline lysis method of Sambrook *et al.* (1989), (Table 2).

Table 2. Protocol for preparation of membranes

Bacterial suspension	Alkaline lysis	Dot blot	DNA binding
200 µl, 0.1 OD_{660nm}	22µl 5M NaOH 60°C, 1 h cool in icewater spin briefly 22µl 5M HCl, 10µl 2M Tris pH 7.5	Bio-Dot Microfiltration Apparatus, Bio-Rad	80°C, 2h

DNA hybridization

Prehybridization and hybridization were carried out at 42°C or 65°C according with the labeling of the DNA and the detection system used.

The hybridization reaction with the probe random primer labeled with digoxigenin d-UTP was detected using the "Genius nonradioactive nucleic acid detection system" (Boehringer Mannheim, Germany) using both color and light (Lumi-Phos 530) reaction.

The hybridizations carried out respectively with the probe labeled with biotin 14 d-ATP and biotin 7 d-ATP were detected according to "PhotoGene" and "BluGene" nucleic acid detection systems respectively (Bethesda Research Laboratory, USA).

Results and discussion

The DNA extracts from AT strains gave positive hybridization in the nonradioactive systems used with the exception of the AT strains CG 113 in the Genius system, and CG 1019 and NCPPB 396 in the BluGene system. In most cases, signal intensity was comparable to that of the control strain DH-1.This

indicates a close relationship between signal and tumorigenicity regardless of the biovar (Fig. 1; Table 1).

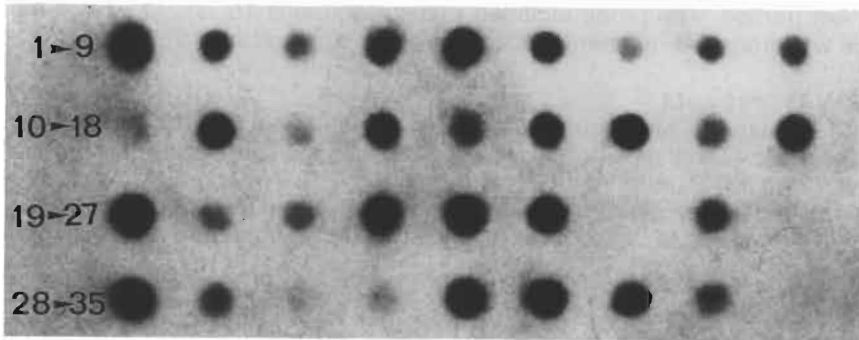

Fig. 1. Bacterial detection using PhotoGene: spots are numbered according to the list of bacterial strains.

A certain degree of hybridization was also detectable with the DNA of the nontumorigenic strains CG1035, K84, CG429 and HLB-2, under standard condition of the system used.

The detection method can influence the results and there are clear differences in the efficiency of the hybridization method. It appears that PhotoGene and Lumi-Phos 530 are the most reliable for the identification of tumorigenic agrobacteria: using these detection systems, all the tumorigenic strains hybridized with the probe.

However, for practical application, positive hybridization signals do not always guarantee the tumorigenic nature of the bacterial strains and this occurred also using the same radioactively labeled probe (Burr *et al.*, 1990). Our results suggest that some of the nontumorigenic strains may carry genes sharing T-DNA homology or an incomplete T-DNA fragment. The pTHE17 DNA fragment carries nopaline catabolic (Noc) gene on its right side (Holsters *et al.*, 1980) and this would explain hybridization with the 200 Kbp plasmid of strain K 84 which carries a Noc function. Another explanation of the fact that hybridization occur with other nontumorigenic strains is that they were originally tumorigenic but lost their oncogenicity, maintaining a nonfunctional T-DNA or common sequences to pTHE17.

The nontumorigenic strains tested may actually be tumorigenic: since AT can infect over 200 species of dycotiledonous plants, they may be host specific and further pathogenicity tests are required (tumorigenicity was checked on kalanchoe, cherry, grapevine, *Nicotiana sp.*, sunflower and tomato; Burr and Bazzi, unpublished). It was also suggested that part of the oncogenic region of the T-DNA might be essential for tumor induction on certain plant species but not on others (Holsters *et al.*, 1980). Therefore, pathogenicity tests cannot be conclusive for determining the presence of this sequence in the bacterial strain tested.

It seems difficult to design a completely specific and reliable method for the detection of tumorigenic agrobacteria. Dong *et al.* (1992) used PCR in comparison with slot blot hybridization and pathogenicity tests but obtained discordant results with respect to the tumorigenicity of some *Agrobacterium* strains. It may not be possible to develop the perfect probe for diagnosis and it is still difficult to establish if is a better strategy to use a large probe like pTHE17 or a smaller one (Burr *et al.*, 1990). Ti-plasmids may vary greatly from strain to strain; one strain may miss genes that are present in other strains, which may still be tumorigenic, but produce tumors on different hosts or with different morphologies.

The usefulness of nonradioactive detection systems, and in particular of the chemiluminescent ones, will be for screening large numbers of strains. Some strains may hybridize but in fact are nontumorigenic.

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Nonradioactive DNA probes for the detection of bacterial ring rot of potato

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A genomic DNA clone of about 1,6 kbp (BB2) has been used as nonradioactive DNA probe to detect Clavibacter michiganense subsp. sepedonicum (Spieck. & Kotth.) Davis *et al.*, the causal agent of bacterial ring rot of potato. Four nonisotopic DNA labelling systems were compared. Three kits were based on incorporation of digoxigenin, biotine modified nucleotides or on detection of sulfonated cystosine. The fourth one was based on peroxidase-labelled DNA probes. Specificity and sensivity levels were variable when labelling systems were compared. Best results have been obtained using digoxigenin detection system. Chromogenic detection using X-phosphate-NBT substrat was sometimes impaired by the brownish color of tissues extracts blotted on filter. The chemioluminescence detection of digoxigenin-labelled BB2 probe increased sensivity level up to 10^4 cfu and signal to noise ratio was better when tissues extracts dot blots were tested.

Digoxigenin -labelled BB2 probe was used to detect bacterial ring rot infestation during summers of 1990 and 1991 in 147 and 232 fields respectively . In 1990, potatoes stems cross-sections were soaked in PBS buffer, then buffer samples were spotted. In 1991, potatoes stems cross-sections were grinded up and clarified homogenate samples were spotted. Relative efficiency of polyclonal ELISA tests and BB2 probe tests were compared using immunofluorescence results as control reference.

Tests	Years	False positives	False negatives
ELISA	1990	5,4%	23,1%
	1991	4,8%	9,0%
Probe	1990	4,8%	1,4%
	1991	6,9%	0,4%

Keywords: Clavibacter michiganense subsp.sepedonicum, DNA probe, potato bacterial ring rot, Solanum tuberosum, nonradioactive labelling.

INTRODUCTION

Clavibacter michiganense subsp. sepedonicum (Spieck. & Kotth.) Davis *et al.* (syn. Corynebacterium sepedonicum (Spieck. and Kotth.) Skapt. and Burkh.) is the causal agent of potato bacterial ring rot. A zero tolerance for bacterial ring rot is required by most potato seed certification agencies. Field inspection to visually detect wilting and others bacterial ring rot symptoms, Gram staining, eggplant seedlings assay and serological techniques are commonly used to find infected plant parts or seed potatoes. However, most of these techniques are not enough specific while others are not suited for large scale screening because they are labor intensive. A plasmid-borned sequence that is also highly repeated in the genome of C. m. subsp. sepedonicum (Cms) (MOGEN & OLESON , 1987; MOGEN *et al.*, 1990; JOHANSEN *et al.*, 1989) and genomic DNA clones III24 and III31 (VERREAULT *et al.*, 1988) were used as DNA probe for specific identification of bacterial ring rot pathogen.

We undertook this work to use a genomic clone, pUC9-III31 as a nonradioactive probe for the detection of C.m. subsp. sepedonicum in field samples. Four nonisotopic DNA labelling systems were compared. Three methods were based on incorporation of digoxigenin (Boehringer Mannheim, Canada), biotine modified nucleotides (Bethesda Research Laboratories, Gaithersburg, MD) or on detection of sulfonated cytosine (ChemoProbe,FMC Bioproducts, Rockland, ME). The fourth one was based on peroxidase-labelled DNA probes (Amersham International). In this report, we describe how subcloning of insert III31 and enzymatic treatments of nylon filters prior to hybridization improved specificity and sensitivity of detection. Efficiency of chromogenic and chemioluminescence detection of digoxigenin-labelled probe was compared using colony blot and potato stem extacts. Relative efficiency of DNA probe and of polyclonal ELISA was also compared using immunofluorescence as a control reference.

MATERIALS & METHODS

Bacterial cultures Bacterial isolates used in this study are listed in table 1. YGM medium (Difco Laboratories, Detroit, MI) was used for all Clavibacter strains and all other bacterial isolates were grown in 523 medium (Kado and Heskett, 1970).

DNA extraction Cultures of C.m.s. were grown 5-7 days, all other cultures were grown 1-2 days. The cultures were harvested by centrifugation at 8500g for 10 minutes, washed in 0,85% NaCl and resuspended in 50 mM Tris-HCl pH 8 containing 10 mM EDTA and lysozyme (2 mg/ml). After 30 minutes of incubation, SDS and proteinase K were added to final concentration of 1% and 0,35 mg/ml respectively and the mixture were incubated overnight or 90 minutes at 50°C. The mixture were extracted with phenol, chloroform, isoamyl alcohol and DNA was precipited with ethanol.

Restriction digest preparation Clone pUC9-III31(15 µg) was digested with restriction enzymes BamH1, EcoR1, HindIII, Pst1, Sac1 (30 µl total volume) as recommended by suppliers of the restriction enzymes (Pharmacia, Montréal, QC., Bethesda Research Laboratories, Gaithersburg, MD). Sufficient incubation times, one hour at 37°C and quantities of enzyme (5 units per µg of DNA were used to assure that complete digests were obtained. The digest DNA was separated by

electrophoresis on 1,4% agarose and restriction fragments were extracted from agarose with the Gene Clean System according to the manufacturer instructions (Bio101, La Jolla,CA).

Potato stem extracts 1 cm cross-sections of potato stem were cut 2 cm above the ground. Potato stem sections were eitheir soaked in PBS buffer for 2 hours at 4°C or grinded up in PBS buffer. Samples were filtered and clarified.

Blotting Serial dilutions of bacteria were prepared with Ringer's solution or with potato stem extracts. Dot blots were made on Gene Screen Plus nylon filters (NEN Research Products,E.I. du Pont de Nemours & Co., Boston, MA) using a blot manifold (Bio-Rad Laboratories, Richmond, CA).

Filter treatment Filters were soaked, colony side up, for 30 minutes at 25°C in 50 mM Tris-HCl pH 8,0 containing 10 mM EDTA and lysozyme (2 mg/ml) and RNAse A (0,1mg/mL). Then filters were transfered in 0,1 M Tris-HCl pH 8,0 containing proteinase K (1 mg/ml) for 20 minutes at 37°C. Then filters were processed by alkaline treatment as described by Grunstein and Hogness (1975).

DNA labelling, hybridization and detection pUC9-III31, insert III31, and restriction fragments of insert III31 were labelled, hybridized , washed and detected according to manufacturer's protocols except when mentionned; Biotin-streptavidin method (Bethesda Research Laboratories), Digoxigenin probe (Boehringer Mannheim), peroxidase-labelled probe (Amersham), and sulfonated probe (Chemiprobe, FMC Bio Products). Probe concentrations during hybridization were 1 µg/ml for biotinylated and sulfonated probe and 150 ng/ml for digoxigenin-labelled probe. X-phosphate and NBT were used as substrats for the alkaline phosphatase enzyme chromogenic detection process. Lumi-Phos 530 was used for the chemioluminescence detection method. Autoradiographic film Kodack X-OMAT AR were used.

Table 1. List of bacterial isolates and their source

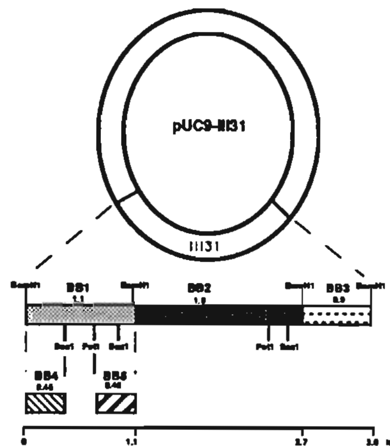
Organisms	Isolate	Source	Organisms	Isolate	Source
Clavibacter michiganense	141	ATCC33111	Clavibacter michiganense	15	J.F.Chauveau
subsp. sepedonicum	142	ATCC33113	subsp. insidiosum	140	ATCC33114
	208	M.Lacroix	C.m. subsp. nebraskense	145	ATCC27822
	a	J.F.Chauveau	Erwinia carotovora	14A	J.F.Chauveau
	6	J.F.Chauveau	subsp. carotovora	20A	J.F.Chauveau
	7	J.F.Chauveau		119	ATCC495
	8	J.F.Chauveau	Erwinia carotovora	24A	J.F.Chauveau
	28	J.F.Chauveau	subsp. atroseptica	116	ATCC4446
	33	J.F.Chauveau	Erwinia amylovora	129	M.Lacroix
	34	J.F.Chauveau		97	ATCC19381
	3	S.H.De Boer		102	ATCC15357
	5	S.H.De Boer		103	ATCC15580
	12	S.H.De Boer		129	M.Lacroix
	15	S.H.De Boer		130	M.Lacroix
	R2	S.H.De Boer		131	M.Lacroix
	R4	S.H.De Boer	Erwinia herbicola	99	ATCC15552
	R6	S.H.De Boer	Pseudomonas syringae	122A	J.F.Chauveau
	R8	S.H.De Boer	Pseudomonas marginalis	162	M.Lacroix
	3R	A.E.Oleson		163	M.Lacroix
	20	A.E.Oleson	Pseudomonas fluorescent	18A	J.F.Chauveau
	106	A.E.Oleson		220	ATCC
Clavibacter michiganense	143	ATCC7429		232	ATCC
subsp. michiganense	144	ATCC14456		233	ATCC
	c	J.F.Chauveau		245	ATCC
	250	J.F.Chauveau			

RESULTS

The detection sensitivity level of labelled DNA probes and peroxidase-labelled DNA probes was reproductible when more than 15 ng of nucleic acids extracts were blotted on nylon membrane. However, false positives were always significantly higher specially when potato tissues extracts were tested. Sulfonated III31 probe and digoxigenin-labelled III31 probe showed similar sensivity. About $1,5x10^5$ of Clavibacter michiganense subsp. sepedonicum (Cms) could be detected. Sulfonated III31 probe was less specific. About $7,5x10^6$ Clavibacter michiganense subsp. michiganense were detected and a weak signal can be observed when more than $2x10^8$ of Erwinia carotovora carotovora were dot blotted. The specificity of digoxigenin-labelled III31 probe was further verified by dot blot assays with several species of Erwinia. Various intensity of non-specific positive signals were observed with when more than $1,8x10^8$ cfu/ml were blotted .

A detailed map of III31 probe that indicates the sites for restrictions enzymes used in this work is shown in Fig.1. Five restrictions fragments of III31 probe (BB1 to BB5) were digoxigenin-labelled and hybridized to Cms and to potato rhizosphere bacterial isolates mentionned above. Among these, only fragment BB2, showed increased specificity. However, digoxigenin-labelled BB2 probe still gave non-specific weak hybridization signal when 10^9 cfu of both Erwinia spp. isolates were blotted .

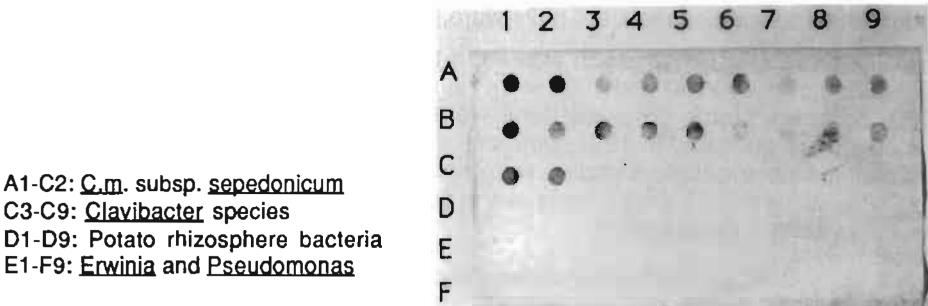
Fig.1 Restriction map of clone pUC9-III31.



Two approaches were investigated to optimize the specificity of the digoxigenin-labelled BB2 probe. One implied that the lack of specificity was caused by proteins and / or polysaccharides released during the nylon membrane process prior to prehybridization step. The other approach relied on increasing the stringency of hybridization and washing steps. Firstly numerous lysis protocols were evaluated. Non-specific signals were still detected when alkaline

treatments (Grunstein and Hogness, 1975) were done at 37°C or 68°C. The specificity could be improved without decreasing the sensivity level, when membranes were soaked in 50 mM Tris-HCl pH 8,0,10 mM EDTA containing lysozyme (2mg/ml) and RNAse A (0,1mg/mL) for 30 minutes at room temperature followed by soaking in buffered proteinase K (0,2 mg/ml) 20 minutes at 37°C. Then, membranes were processed by alkaline treatment.

Fig.2 Specific identification of C.m.subsp. sepedonicum with BB2 digoxigenin-labelled probe. 9 x10^9 cfu of each bacterial isolates were blotted as described.



A1-C2: C.m. subsp. sepedonicum
C3-C9: Clavibacter species
D1-D9: Potato rhizosphere bacteria
E1-F9: Erwinia and Pseudomonas

	1	2	3	4	5	6	7	8	9
A	a	6	7	8	28	33	34	5	3R
B	15	106	R2	R4	R6	R8	12	3	142
C	141	208	143	144	145	c	250	15	140
D	172	174	178	179	186	187	190	191	192
E	47	STR2	39	99	100	14A	20A	119	24A
F	116	122A	162	163	18A	220	232	233	245

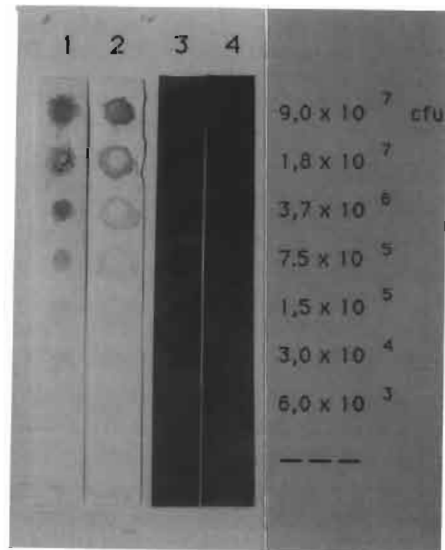


Fig.3 Detection of C.m. subsp. sepedonicum in Ringer's solution and in potato stem extracts using BB2 digoxigenin-labelled probe with chromogenic or chemioluminescence detection method.
Five fold dilutions of C.m.s.#7 in Ringer's solution (1, 3) and in potato stem extracts (2, 4) were spotted. Chromogenic (1, 2) and chemioluminescence (3, 4) detection were done using substrat X-phosphate-NBT or Lumi-Phos 530 respectively.

Secondly, the specificity was increased when hybridization and washing were done at 76°C instead of 68°C or 72°C or any combinations of these two temperatures. Digoxigenin-labelled BB2 probe under such improved conditions showed high specificity (Fig.2). At least 2,4 x10^4 cfu of C.m. subsp. sepedonicum could be detected when bacteria were diluted in Ringer's solution, whereas 1.2 x 10^5 cfu of C.m. subsp. sepedonicum were detected when potato stem extracts were tested (Fig.3).

Chromogenic detection using X-phosphate-NBT substrat was sometimes impaired by the brownish color of tissues extracts blotted on filter. The chemioluminescence detection of digoxigenin-labelled BB2 probe increased sensitivity level up to 10^4 cfu and signal to noise ratio of BB2 probe was better when potato tissues extracts dot blots were tested (Fig. 3).

Digoxigenin labelled BB2 probe was used to detect bacterial ring rot infestation during summers of 1990 and 1991 in 147 and 232 fields respectively (table 2). In 1990, potatoes stems cross-sections were soaked in PBS buffer, then buffer samples were spotted. In 1991, potatoes stems cross-sections were grinded up and clarified homogenate samples were spotted. Relative efficiency of polyclonal ELISA tests and BB2 probe tests were compared using immunofluorescence results as control reference.

Table 2. Comparaison of relative efficiency of polyclonal ELISA and BB2 probe.

Tests	Years	False positives	False negatives
ELISA	1990	5,4%	23,1%
	1991	4,8%	9,0%
Probe	1990	4,8%	1,4%
	1991	6,9%	0,4%

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Characterization of *Agrobacterium* biotypes by RFLP analysis of PCR amplified 16S gene

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Agrobacterium strains belong to three biotypes determined on the basis of biochemical characteristics, which may correspond to three different species. We have developed a PCR/RFLP method on the 16S gene of the ribosomal DNA operon to rapidly and accurately discriminate the three *Agrobacterium* biotypes. The study was done with 35 strains belonging to the three biotypes isolated from various host-plants and geographical origins. Universal primers were used to amplify the 1500pb 16S rDNA gene by Polymerase Chain Reaction (P.C.R.) The amplified 16S gene DNAs were digested by three restriction endonucleases with tetranucleotide recognition and cleavage sites. The 16S rDNA restriction fragment length (R.F.L.P) patterns allowed us to discriminate the three biotypes. This PCR/RFLP strategy is thought to be applicable to determine rapidly the biotype of *Agrobacterium* isolates.

Key words: *Agrobacterium*, biotype, PCR/RFLP, 16S gene.

According to the Bergey's manual of Systematic bacteriology (KERSTERS & DE LEY., 1984), *Agrobacterium* still comprises different species on the basis of biochemical and phytopathological properties. This last feature seems unsuitable since it is now well-known that phytopathogenicity is due to plasmids Ti or Ri (for review see WINANS *et al.* 1992), which are transferable genomic elements not always present in *Agrobacterium* strains. Extensive studies were performed on the basis of biochemical characteristics (KERSTERS *et al.* 1973, HOLMES & ROBERTS, 1981) and DNA/DNA hybridization (DE LEY *et al.* 1973, POPOFF *et al.* 1984) and led to group *Agrobacterium* strains in three biotypes. The different biotypes correspond to the different species of *Agrobacterium*, suggested by

HOLMES *et al* (1981), and now definitively accepted for biotype3 strains, that are grouped into species *Agrobacterium vitis* (OPHEL & KERR, 1990).The biotype characterization is time consumming since it usually requires the use of biochemical tests. In order to obtain a faster and accurate determination of *Agrobacterium* species, we developed a test based on the occurence of biotype specific variability in the ribosomal 16S gene. For this purpose, we characterized each strain by RFLP analysis of PCR amplified 16S gene (as described by NAVARRO *et al.* 1992). This paper shows the suitability of the 16S gene PCR/RFLP to rapidly and accurately distinguish the three main *Agrobacterium* biotypes.

MATERIALS AND METHODS

Bacterial strains. The 35 strains of *Agrobacterium sp.* (SMITH & TOWNSEND) CON. used in this study were isolated from various host-plants and geographical origins. They were assigned to three biotypes as follows: biotype1 (296, 354, C58, 1904, 2407, 2410, 2411, 2414, 2458, 2177, 2516, 2517, 2518, CG1028), biotype2 (450, 1317, 1804, 1905, 1936, 1961, 1962, 2178, 2417, 2418, 2419, 2519, 2520, CG1029, CG1030, CG1031) and biotype3 (2512, 2618, 2620, 2621, 2622).

PCR amplification. Amplification reactions were performed according to the protocol of MULLIS & FALOONA (1987) with primers flanking the 16S gene of the ribosomal DNA operon. Primers FPGS6 (5' TGCGGCTGGATCCCCTCCTT 3') and FPGS1500' (5' CCATGTTGTTTGCTAGCCAG 3') hybridize respectively at 8pb and 1499pb from the beginning of the 16S gene according to the sequence published by YANG *et al.* (1988).

The amplification mix contained 0,1μM of each primer, 20μM of each dNTP, 1X reaction buffer (10mM Tris-HCl pH8,3, 50mM KCl, 1,5mM MgCl2, 0,01% gelatin) and 2,5 units of Taq polymerase (Gibco BRL). Amplification was done by 35 cycles of denaturation (1 minute at 95°C), hybridization (1 minute at 55°C) and extension (2 minutes at 72°C).

DNA digestion. Restriction digestions were performed in 15μl volume, following conditions recommended by suppliers. About 5μl of amplified DNA were digested with *NdeII*, *HaeIII* and *TaqI* (Appligene, Gibco BRL, Boerhinger). Digested DNA was separated on a 3% Nusieve agarose gel and run at 80mV for 3h30.

Map of 16S gene. To construct the map of standard biotype1 strain, we looked for typical four base recognition sites of endonucleases *NdeII*, *TaqI* and *HaeIII* in the published sequence (WANG *et al.*, 1988) (Fig.1). Profiles obtained with other

strains were compared to the standard map and polymorphic sites were indicated accordingly.

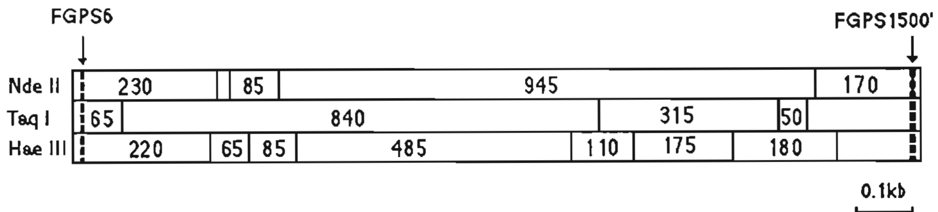

Fig.1 Restriction map of the 16S gene of strain C58 (adapted from Wang *et al.*)

RESULTS and DISCUSSION

Using primers FPGS6 and FPGS1500', amplification were obtained with all the 35 tested strains. As expected from the published sequence (WANG *et al.* 1992) the length of the amplified DNA was approximatively 1500pb. Amplified DNA was then digested with *NdeII, TaqI* and *HaeIII.* DNA fragments length, obtained by PCR/RFLP of standard biotype1 strain C58, were identical to those expected from the sequence analysis (Fig.1). This indicates that amplified DNAs correspond to the 16S gene.

The comparison of the 35 profiles showed the occurence of polymorphic restriction sites with all three tested endonucleases. This allowed us to construct different 16S gene maps. With *NdeII* and *TaqI,* there were respectively two and one restriction sites, which permitted to distinguish all biotype2 strains from strains belonging to biotype1 or 3 (Fig.2). With *HaeIII* the polymorphic sites allowed us to discriminate: the three biotypes and a supplementary site which separates strains belonging to biotype1 in two groups (C58, 2410, 2517, 354, 2516, 2177) and (1904, 2518, 2458, 2407, 2411, 2414, 296) (Fig.2).

PCR/RFLP performed in the 16S gene permitted the recognition of taxonomic lineages, the biotypes (KEANE *et al.* 1970), which are now considered as corresponding to species (OPHEL & KERR, 1990). This method also confirmed the heterogeneity of the biotype1 cluster, this is in agreement with DE LEY *et al.* (1973) and POPOFF *et al.* (1984).

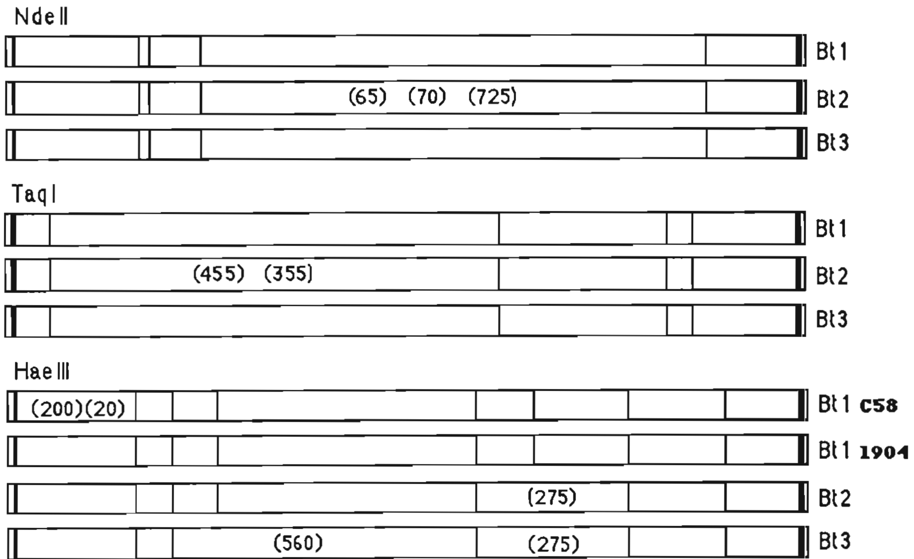

Fig.2 Restriction maps of the 16S amplified DNA digested with *NdeII, TaqI, HaeIII*

(only molecular weight of bands different from the standard strain were indicated, primers locations are indicated by black lines)

The occurrence of mutations in the 16S gene, as determined by sequencing (NAZARET *et al.*, 1991), has been reported between bacteria belonging to different genomic species. Our results agree since biotype3 is a genomic species called *Agrobacterium vitis*, different from biotypes1 and 2. Furthermore, our findings confirm the hypothesis of HOLMES *et al.* (1981), that biotypes1 and 2 are in fact two species of the genus *Agrobacterium*.

These results showed the suitability of the PCR/RFLP of 16S gene to rapidly identify the biotype (i.e. species) of *Agrobacterium* isolates. It will be useful to assess the biotype status of strains with undetermined or intermediate biotype (BELL & RAMEY, 1991 , BOUZAR & MOORE, 1987).

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The use of a DNA probe and PCR for the detection of *Pseudomonas syringae* pv. *pisi* in pea seed

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ABSTRACT

A cloned DNA probe developed in Denmark (Rasmussen and Wulff, 1990) was extensively screened for its specificity to *Pseudomonas syringae* pv. *pisi.* A range of isolates of the main UK races of the pathogen with co-isolated non-pathogenic organisms from UK pea seed samples and other phytopathogenic bacteria were tested. Partial sequencing of this DNA fragment provided primers for use in the polymerase chain reaction (PCR). A seed test based on a PCR detection method using these primers was developed (Rasmussen and Wulff, 1991). It was optimised and evaluated using seed-soak liquors obtained from UK pea seed samples which were tested in parallel with traditional techniques. The results of the screening and the comparison of seed testing techniques are presented.

Keywords: *Pseudomonas syringae* pv. *pisi,* DNA probes, PCR.

INTRODUCTION

In the UK control of the seed-borne pathogen *Pseudomonas syringae* pv.*pisi* requires testing of large numbers of seed samples. The current seed test (figure 1) is sensitive and accurate but lengthy, time-consuming and expensive. This report describes recent results in the development of a new seed test based on a DNA probe and PCR.

METHODS

DNA probe :

The development and initial screening of pPSP68 was described by RASMUSSEN and WULFF (1990). This fragment was used as a probe and was labelled with [α-32P]dCTP using the Amersham Multiprime system according to the manufacturer's instructions.

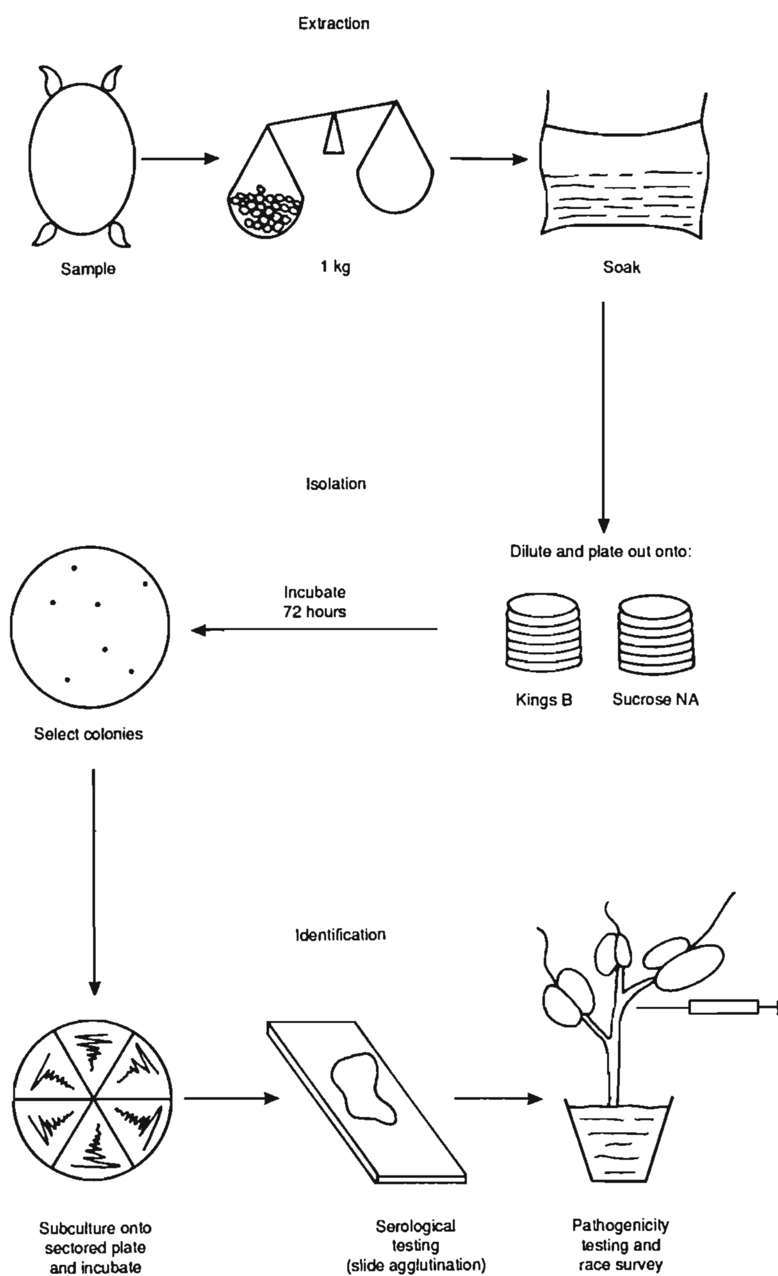

Figure.1 A typical seed test for pea bacterial blight.

Bacterial isolates:

In order to confirm the specificity of the DNA probe 95 bacterial isolates were tested. The 7 races of *Pseudomonas syringae* pv. *pisi* used as control isolates were obtained from Dr J.D. Taylor as was the isolate of *Pseudomonas syringae* pv.*syringae*. The isolate of *Xanthomonas campestris* pv. *campestris* was NCPPB B9051. 53 other isolates of unknown identity were obtained at NIAB from pea seed samples and represent the background bacterial microflora found in seed tests. Of these unknowns 27 resembled *P.s.pisi* in their colony morphology and 13 of these 27 were tested with an antiserum raised against *P.s.pisi*; 10 gave a positive result. The two *Erwinia* isolates were also obtained at NIAB. 24 *P.s.pisi* isolates obtained from tests on pea seed at NIAB were also included. All isolates were checked for pathogenicity against Kelvedon Wonder pea seedlings to confirm their identity. Only *P.s. pisi* gives typical water-soaked lesions on this host.

Dot-blotting:

Bacterial isolates were grown in yeast tryptone broth at 25°C to late log phase and cell concentrations were adjusted to be approximately equal in all cultures. The cultures were then boiled for two minutes and chilled on ice before 100μl of each was loaded onto a Millipore dot-blotter containing GeneScreen Plus (Du Pont) membrane. After blotting the membrane was allowed to surface dry and then microwaved at full power in a 500watt microwave oven for 3 minutes and 15 seconds.

DNA hybridisation:

Prehybridisation was done essentially according to Du Pont recommendations for their membrane without dextran sulphate but with the addition of Denhardt's and single-stranded salmon sperm DNA. Membranes were prehybridised for a minimum of 2 hours. The labelled probe was added and hybridisation took place overnight at 65°C. Following this the membrane was washed twice in 2xSSC at 65°C for 15 minutes, once in 2x SSC and 0.1% SDS at 65°C for 30 minutes and finally with a high stringency 0.1x SSC wash at 65°C for 10 minutes. The blots were autoradiographed at -76°C with intensifying screens for at least 24 hours.

Seed testing using PCR:

Further develoment of a PCR-based seed test reported by Rasmussen and Wulff (1991) is reported here. 1kg of seed from 10 different pea seed samples was soaked

in 3L of filtered water at 4°C for 24 hours. The liquors were sampled at 6 hours and again after 24 hours. For each of these samples 1-5 ml was taken, spun for 5 minutes at 20,000 x g, the supernatant was removed and the pellet resuspended in 100µl of TE buffer. This was repeated before boiling for 5 minutes and sampling for PCR. The results of this were compared with the results of a standard seed test.

Amplification conditions:
PCR was conducted in 100µl volumes using 5µl of sample in a standard reaction mixture for 35 cycles of 94°C for 1 minute, 68°C for 1 minute and 72°C for 2 minutes. The primers used were external to the pPSP68 fragment.

RESULTS AND DISCUSSION

Figures 2a and 2b show the autoradiographs of the probed dot-blots. These results are summarised in Table 1 beside the results from the pathogenicity testing of the various isolates used in the dot-blotting.

TABLE 1
Results of probing with pPSP68 the bacterial isolates shown below and of pathogenicity testing against Kelvedon Wonder seedlings.
a = unidentified isolate of bacterial microflora found on pea seed.

DOT BLOT 1 (Fig.2a)

ISOLATE	PROBE	PATHOGENICITY	WELL POSITION	ISOLATE	PROBE	PATHOGENICITY	WELL POSITION
P.s.pisi race 1	+	+	1A	*E.carotovora sub sp atroseptica*	-	-	3C
P.s.pisi race 2	+	+	2A	a	-	-	4C
P.s.pisi race 3	+	+	3A	a	-	-	5C
P.s.pisi race 4	+	+	4A	a	-	-	6C
P.s.pisi race 5	+	+	5A	a	-	-	1D
P.s.pisi race 6	+	+	6A	Broth control	-	N/A	2D
P.s.pisi race 7	+	+	1B	a	-	-	3D
a	-	-	2B	a	-	-	4D
P.s.pisi	+	+	3B	a	-	-	5D
P.s.pisi	+	+	4B	a	-	-	6D
P.s.pisi	+	+	5B	a	+	-	1E
P.s.pisi	+	+	6B	a	+	-	2E
P.s.syringae	-	-	1C	a	-	-	3E
E.rhapontici	-	-	2C	a	-	-	4E

ISOLATE	PROBE	PATHOGENICITY	WELL POSITION	ISOLATE	PROBE	PATHOGENICITY	WELL POSITION
X.c.campestris	-	-	5E	*P.s.pisi*	+	+	3G
a	-	-	6E	*P.s.pisi*	+	+	4G
P.s.pisi	+	+	1F	*P.s.pisi*	+	+	5G
P.s.pisi	+	+	2F	*P.s.pisi*	+	+	6G
P.s.pisi	+	+	3F	*P.s.pisi*	+	+	1H
P.s.pisi	+	+	4F	*P.s.pisi*	+	+	2H
P.s.pisi	+	+	5F	*P.s.pisi*	+	+	3H
P.s.pisi	+	+	6F	*P.s.pisi*	+	+	4H
P.s.pisi	+	+	1G	*P.s.pisi*	+	+	5H
P.s.pisi	+	+	2G	*P.s.pisi*	+	+	6H

DOT BLOT 2 (Fig.2b)

ISOLATE	PROBE	PATHOGENICITY	WELL POSITION	ISOLATE	PROBE	PATHOGENICITY	WELL POSITION
P.s.pisi race 1	+	+	1A	a	-	-	1E
P.s.pisi race 2	+	+	2A	a	-	-	2E
P.s.pisi race 3	+	+	3A	a	-	-	3E
P.s.pisi race 4	+	+	4A	a	-	-	4E
P.s.pisi race 5	+	+	5A	a	-	-	5E
P.s.pisi race 6	+	+	6A	a	-	-	6E
P.s.pisi race 7	+	+	1B	a	-	-	1F
P.s.pisi	+	+	2B	a	-	-	2F
P.s.pisi	+	+	3B	a	-	-	3F
a	-	-	4B	a	-	-	4F
a	-	-	5B	a	-	-	5F
a	-	-	6B	a	-	-	6F
a	-	-	1C	a	-	-	1G
a	-	-	2C	a	-	-	2G
a	-	-	3C	a	-	-	3G
a	-	-	4C	a	-	-	4G
a	-	-	5C	a	-	-	5G
a	-	-	6C	a	-	-	6G
a	-	-	1D	a	-	-	1H
a	-	-	2D	a	-	-	2H
a	-	-	3D	a	-	-	3H
a	-	-	4D	a	-	-	4H
a	-	-	5D	a	-	-	5H
a	-	-	6D	a	-	-	6H

TABLE 2

The results of PCR on seed-soak liquors sampled after 6 hours and 24 hours compared with the standard seed test.

SEED SAMPLE	PCR		STANDARD TEST	SEED SAMPLE	PCR		STANDARD TEST
	6h	24h			6h	24h	
445	+	-	+	1009	-	-	-
618	+	-	+	1023	-	-	-
646	+	+	+	1026	-	-	-
652	+	+	+	1027	-	-	-
657	-	-	+	1028	-	-	-

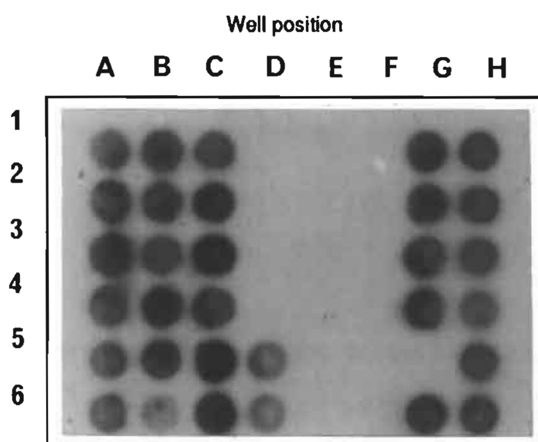

Fig. 2a

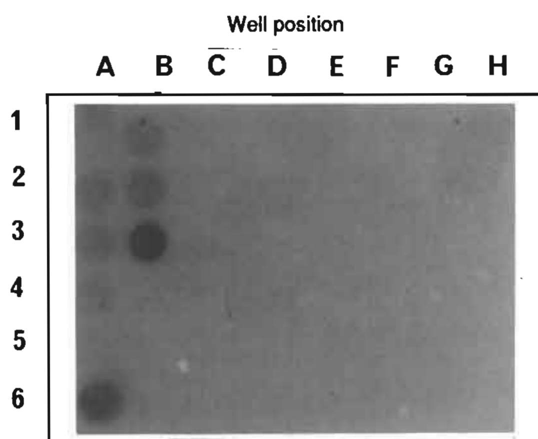

Fig. 2b

Figures 2a and 2b
Autoradiographs of dot-blots probed with pPSP68

Table 1 shows the specificity of the probe pPSP68. Only in two cases did this probe hybridise to bacterial isolates which were non-pathogenic on Kelvedon Wonder pea seedlings, the indicator plant for the disease caused by *P.s.pisi.* These bacteria appear to be very similar to *P.s.pisi,* producing typical colonies on Kings B agar, levan colonies on sucrose nutrient agar and both react with an antiserum raised against *P.s.pisi* (results not shown). Further investigations of these isolates are underway as the possibility that they are non-pathogenic strains of *P.s.pisi* cannot be discounted. No false negative results were found, confirming the preliminary screening by Rasmussen and Wulff (1990).Results from the PCR experiments were more equivocal (Table 2). There was a clear effect of soaking time on the PCR with 6h giving better results than 24h. This agrees with earlier results (Rasmussen and Wulff,1991) where this effect was attributed to the release of unknown inhibitory compounds from the pea seed interfering with the PCR reaction. Not only does this effect appear to increase with soaking time but also varies between pea cultivars (data not shown). This may explain the false negative result given with seed sample 657. Further work is underway in order to identify the causes of variability in the results of PCR on the seed-soak liquors in order to optimise reaction conditions.

CONCLUSIONS

The probe pPSP68 is of clear value in identifying *P.s.pisi* from other bacterial isolates found on pea seed. This utility is being exploited in the development of a seed test based on colony hybridisation, although a final pathogenicity test may be required to avoid the possibility of falsely positive results. Tests based on DNA probes for the identification and detection of phytopathogenic bacteria are now becoming more widespread although as yet there are few reports of their routine use (Rasmussen and Reeves, 1992). The efficiency of the newly developed test will be improved in the medium term by further development of PCR detection and identification of the pathogen.

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Detection of *Pseudomonas syringae* pv. *glycinea* in soybean seed lots intended for import to Greece in 1990

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ABSTRACT

A method was developed for the detection of *Pseudomonas syringae* pv. *glycinea* (Psg) in soybean seed lots, including : (a) sampling of 1kg seed (approx. 5000 seeds) per seed lot of 20 ton, (b) direct isolation of Psg from 20-24 h seed washings on nutrient sucrose agar medium supplemented with cephalexin and cycloheximide and (c) identification of suspect colonies by a few biochemical and two pathogenicity tests. Twenty out of eighty eight samples of soybean seed lots from 34 varieties were found to be infected by Psg. The number of infected subsamples within each sample (5 subsamples/sample) was 5 in 60% of the infected samples, 4 in 5%, 3 in 5%, 2 in 20% and 1 in 10% of the infected samples. The population of Psg in the seed washings of infected subsamples was ranging from $1x10^1$ to $1x10^6$ cfu/ml, but the mean was about $1x10^5$ cfu/ml. The detection treshold of the method was 3 cfu/ml.

The population of saprophytic bacteria recovered from all samples was ranging from $1x10^1$ to $7x10^4$ cfu/ml. Of the other bacteria infecting soybeans, only *Pseudomonas syringae* pv. *syringae* was defected on one variety.

KEYWORDS

Detection, *Pseudomonas syringae* pv. *glycinea*, soybean, seed lot.

INTRODUCTION

Pseudomonas syringae pv. *glycinea* (Psg), the soybean blight bacterium, has been spread worlwide (SINCLAIR & SHURTLEFF, 1982) with infected seed and is considered the most important among all prokaryotes infecting soybeans (CHO & YOO, 1977 ; KENNEDY & ALCORN, 1980 ; WILLIAMS & NYVALL, 1980).

Since visual inspection of the seed is unreliable, special laboratory methods are required to identify seed lots infected by Psg. KENNEDY (1969) detected Psg by inoculating seedlings with a suspension made from crushed seeds, while NICHOLSON & SINCLAIR (1971) placed surface-disinfected seeds on agar media and observed growth of Psg. PARASHAR & LEBEN (1972) detected seed-borne Psg by wounding the cotyledons of partly germinated seeds and providing water saturated air into which seedlings merged. In this paper we describe a new laboratory method and provide detailed results of the laboratory testing of 88 seed samples from 34 soybean varieties intended for import to Greece in 1990.

MATERIALS AND METHODS

Bacterial cultures

Isolate NCPPB 2411 of Psg grown on NAG medium (nutrient broth Oxoid 1.3%, Agar Oxoid No 3 1.2%, glycerol 2% w/v) was used as positive control in the various tests.

Seed material

Seed material used in this study was from 34 soybean varieties (*Glycine max* (L.) Merill) (cvs Williams, Gemma, Weber, Arizona, Carolina, Katai, A-4595, a-3205, A-3733, 8841, 8927, 3701812, 8839, 89299, 89101, 89102, S-3636, S-4230, Hodgson, Fransoy, Kingsoy, Smeralda, Zaffira, Apache, Baron, Crusader, Aura, Birch, Valerma, Halaya, PA, MA, VA, BO) intended for import to Greece in 1990. Eighty eight samples of 1 kg seeds (approx. 5000 seeds) were taken from every lot (of 20 ton) of each variety.

Each sample was divided in 5 subsamples (5 x 1000 seeds) (5 x 150-200 g) each of which was placed in 500 ml (150 g seeds) or 600 ml (200 g seeds) sterile deionized water (SDW) contained in 1 l sterile glass conical flask, at 4-6°C for 20-24 h.

Isolation of Psg

Each flask was shaken vigorously and three serial 10-fold dilutions of the seed extract were made in SDW. An aliquot of 200 μl from each dilution and the undiluted extract was plated on to each of two plates of NAS medium (nutrient broth Oxoid 1.3%, Agar Oxoid No 3 1.2%, sucrose 5% w/v) supplemented with cephalexin (20 μg/ml) and cycloheximide (250 μg/ml). Plates were incubated inverted at 25°C for 2-3 days.

When levan producing colonies were seen the subsample was considered as suspect and 5 at least colonies per subsample were picked up and transferred on NA medium (nutrient broth Oxoid 1.3%, agar Oxoid No 3 1.2% w/v) slants for further testing.

Biochemical tests

Oxidase production, fluorescent pigment production (under UV light at 254 nm), oxidative/fermentative glucose metabolism, aesculin and gelatin hydrolysis and acid production from inositol and sorbitol were tested as described earlier (ALIVIZATOS, 1979).

Pathogenicity tests

Hypersensitive reaction (HR) on tobacco leaves was tested as described by KLEMENT (1963). Pathogenicity on soybeans was tested mainly on cv. Katai or Arizona : (a) as described by LELLIOTT and STEAD (1987) and (b) by pricking pregerminated seeds, soaking in the bacterial suspension (5 x 10^7 cfu/ml) for 15 min and planting in sterile soil at 25°C.

RESULTS AND DISCUSSION

Bacterial isolates

Eighty three subsamples out of 440 (21 samples out of 88) from 14 varieties produced levan colonies and 415 oxidase negative isolates were obtained on NA medium (Table 1). All other varieties were free of Psg.

Characterization of isolates

The results on the biochemical and pathogenicity tests identified the 410 isolates from the 13 varieties (Weber, Fransoy, Gemma, Kingsoy, Hogdson, 3701812, S-3636, PA, MA, VA, 8839, Aura, Apache) as *P.s.* pv. *glycinea* (Psg) and the 5 isolates from the variety Williams as *Pseudomonas syringae* pv. *syringae* (Pss) (Table 1).

Recovered population of Psg and saprophytes

The mean number of cfu of Psg recovered from the seed extract of the subsamples of each variety was ranging from 6.5 x 10^2 to 1 x 10^6 cfu/ml, but the lowest defected number in a subsample was 10 cfu/ml (Table 2). The population of saprophytic bacteria in the subsamples of all samples ranged from 10 to 7 x 10^4 cfu/ml. Also all subsamples free of Psg showed similar populations of saprophytes.

Table 1. Biochemical and pathogenicity tests of isolates from soybean seeds.

Variety	Number of Isolates	Levan	Oxidase	Fluorescent pigment (254 nm)	Hydrolysis		O/F test	Acid from		HR	Water soaked lesions on soybeans	Bacterium
					Aesculin	Gelatin		Sorbitol	Inositol			
Weber	175	+	-	+	-	-	0	-	+	+	+	Psg
Fransoy	40	+	-	+	-	-	0	-	+	+	+	Psg
Gemma	30	+	-	+	-	-	0	-	+	+	+	Psg
Kingsoy	25	+	-	+	-	-	0	-	+	+	+	Psg
Hogdson	25	+	-	+	-	-	0	-	+	+	+	Psg
3701812	25	+	-	+	-	-	0	-	+	+	+	Psg
S-3636	25	+	-	+	-	-	0	-	+	+	+	Psg
PA	25	+	-	+	-	-	0	-	+	+	+	Psg
MA	10	+	-	+	-	-	0	-	+	+	+	Psg
8839	10	+	-	+	-	-	0	-	+	+	+	Psg
Aura	10	+	-	+	-	-	0	-	+	+	+	Psg
VA	5	+	-	+	-	-	0	-	+	+	+	Psg
Apache	5	+	-	+	-	-	0	-	+	+	+	Psg
Williams	5	+	-	+	+	+	0	+	+	+	-	Pss
NCPPB 2411	1	+	-	+	-	-	0	-	+	+	+	Psg

Table 2 : Number of subsamples found infected within infected samples and recovered population of Psg in the seed extracts

Infected Varieties	Samples		Grouping of samples according to infected subsamples					Mean [1] cfu/ml of seed extract
	examined	infected	1	2	3	4	5	
Weber	10	7					7	1.6×10^5
Fransoy	2	1			1			5.0×10^4
Gemma	3	2		1		1		2.5×10^5
Kingsoy	1	1					1	2.0×10^4
Hogdson	1	1					1	1.6×10^4
3701812	1	1					1	2.1×10^5
S-3636	1	1					1	1.0×10^6
PA	1	1					1	2.3×10^4
MA	1	1		1				4.2×10^4
8839	1	1		1				6.5×10^2
Aura	1	1		1				2.1×10^3
VA	1	1	1					1.7×10^5
Apache	1	1	1					7.5×10^3
Total	25	20	2	4	1	1	12	

1 Mean of all infected subsamples, cfu : colony forming units

Table 2 also shows that the number of infected subsamples within each sample was 5 in 60% of the infected samples, 4 in 5%, 3 in 5%, 2 in 20% and 1 in 10% of the infected samples.

DISCUSSION

The described method of direct isolation of Psg from soybean seed extracts detected as low as 10 cfu/ml (5×10^3 cfu/1000 seeds) on NAS medium. This simple medium supplemented with cephalexin (CE) and cycloheximide (Cy) is recommended for the direct isolation of Psg. In a recent study (ALIVIZATOS, 1992, unpublished data) NAS (+Ce, Cy) medium was found to be superior than King's B and the selective M71 (LEBEN, 1972). Boric acid contained in M71 was slightly inhibitory to Psg. The biochemical tests used were the minimum most useful ones to distinguish Psg from other *P. syringae* pathovars infecting soybeans. An agreement was found between biochemical and pathogenicity tests.

Soybean cotyledons inoculated with the method of LELLIOTT and STEAD (1987) showed after 3 days (at 20-23°C) dark brown to black necrosis surrounded (in most cases) by a halo, but only water-soaked lesions with bacterial exudate when inoculated with the second method. The later method is most preferable because of the characteristic symptoms, but it may take longer (4-5 days).

Our method requires 7 days for a positive diagnosis and is superior than the methods described by KENNEDY (1969), NICHOLSON & SINCLAIR (1971) and PARASHAR & LEBEN (1972). Its sensitivity can be increased by centrifuging (10000 g/6°C/15 min) a volume (i.e. 40 ml) of the seed extract, resuspending the pellet in 1 ml SDW and plating serial 10-fold dilutions.

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Physiological and pathological characterization of a non-fluorescent pathovar of *Pseudomonas syringae* isolated from coriander

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ABSTRACT

Since 1987 a hitherto undescribed bacterial pathogen has regulary been isolated from diseased coriander fields in Germany. Infected tissue always contained great quantities of rod-shaped motile bacteria with three polar flagella.
Presumptive characterization and LOPAT tests revealed that the non-fluorescent pathogen belongs to group 1 a of *Pseudomonas syringae*.
Host range tests showed that only few *Umbelliferae* could be affected by the pathogen. It was concluded that the causal organism should be classified as a new pathovar of *Pseudomonas syringae*.

INTRODUCTION

A bacterial disease of coriander (*Coriandrum sativum* L.) causes great yield-losses in West-Germany since several years.
Symptoms appear on leaves, petioles, stems and umbels. On young plants infected leaves first show small water-soaked lesions which later change into red-brown necrotic spots. Severely infected leaves and petioles become black and soft. Attacked stems show elongated brownish lesions surrounded by small, dark green, water-soaked haloes. In the field the disease was frequently observed during blossom and ripening stage, with flowerstands turning to a brownish-mauve colour. On young seeds water-soaked areas are typical symptoms. Later on the seeds discolour black and shrivel.
Similar diseases of coriander, called umbel blight, seed decay or bacteriosis of coriander, were described in Poland (Godlewska-Lipowa, 1966), Hungary (Nemeth

et al., 1969), Great Britain (Taylor and Dudley, 1980), East-Germany (Plescher, 1983), Mexico (Perez et al., 1990) and in California (Cooksey et al., 1991). In West-Germany Mavridis et al. (1989) identified a *Pseudomonas* species as the causal agent of the disease. The isolated bacteria were similar to strains from England, Mexico and California. The aim of this study was to further characterize the pathogen which appeared to be an undescribed pathovar of the *Pseudomonas syringae* group.

ISOLATION

Infected tissues always contained large numbers of rod-shaped motile bacteria with mostly three polar flagella (Fig. 1). The isolations usually resulted in circular, non-fluorescent, greyish colonies on King's medium B agar (King et al., 1954). After growth for more than seven days the colonies showed a slight blue fluorescence under UV-light (360 nm), but no diffusible fluorescent pigment was secreted into the surrounding agar.

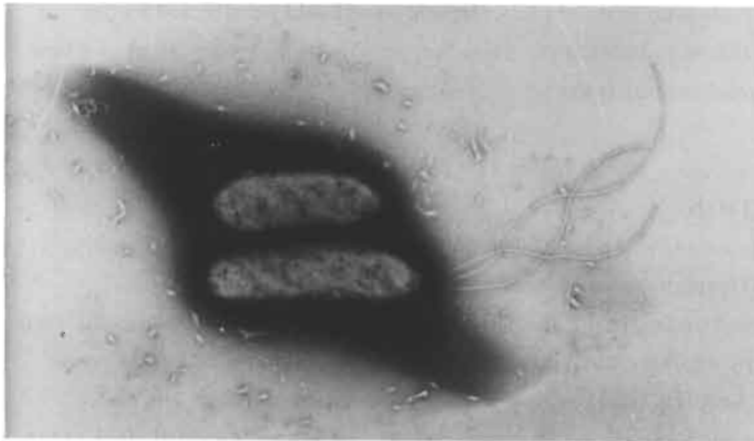

Figure 1: EM-photo of a typical *Pseudomonas syringae* isolate from coriander with monopolar flagellation

PATHOGENICITY

All 120 isolates obtained from 1987 to 1991 in Northern Germany induced a hypersensitive reaction on tobacco leaves. Pathogenicity tests on coriander were carried out with all strains. Coriander plants (*Coriandrum sativum* var. *microcarpum*, "Corry") were inoculated with bacterial suspension (approx. 10^4

cfu x ml^{-1}) from freshly grown cultures. The umbels were sprayed during flowering until little droplets become visible. Young leaves were inoculated by infiltration into the adaxial surface using a glass atomizer. The treated plants were incubated for 14 days at 20 °C and high RH (approx. 50-70 %) in the glass house.
All strains induced water-soaked lesions both on young fruits and on leaves 7 days after inoculation. After 14 days the umbels blackened and the fruits shrivelled. Later on filamentous bacterial exudates developed. The leaves showed little red-brown spots surrounded by small water-soaked haloes.
The inoculated bacteria could be re-isolated from affected tissue in all instances.

CHARACTERIZATION OF THE PATHOGEN

All 120 isolates were Gram-negative, rod-shaped, motile and strictly aerobic. On YDC-agar they formed pale-white, circular, convex colonies with an entire edge. Tests for LOPAT reactions (Lelliott et al., 1966) showed that the isolates were positive for levan production and tobacco hypersensitivity reaction, but negative for oxidase and arginine dihydrolase.
Most of the isolates did not cause rot of potato slices, but 20% of the strains consistently showed a weak reaction.
Further tests were carried out with nine representative isolates from coriander, and one identical strain isolated from wild carrot.
All isolates reacted negatively for 2-keto-gluconate production, egg yolk reaction, nitrate reduction and arbutin hydrolysis.
The isolates neither accumulated nor utilized poly-ß-hydroxybutyrate.
Pectolytic activity was detected by growth on Hildebrand's medium C (pH 8.5) (1971), whereas the strains reacted indifferently on medium B (pH 7). No gel-pitting resulted after growth on medium with low pH.
All tested isolates hydrolysed Tween 80 but did not liquify gelatine.
Eight of ten isolates showed ice-nucleation activity at - 5 °C. The strains were able to grow between 4 °C and 34 °C. The thermal death-point was estimated between 51 - 52 °C.
The utilization of different carbon sources was investigated on the medium of Ayers et al. (1919) containing 1% (w/v) of the tested carbohydrates.
The bacteria did not grow on adonitol, D-arabinose, benzoate, erythritol, geraniol, homoserine, L-lactate, L-rhamnose, L-tartrate and trigonelline. A very slow and weak growth was observed on quinate and cellobiose. The substrates betaine, glucose, inositol, D-mannitol, sorbitol, sucrose and D-tartrate were well utilized. These results correspond to those obtained with the Biolog system (von Kietzell et al., 1992).

Tests for syringomycin production on PDA did not result in inhibition of *Geotrichum candidum*.

HOST RANGE

Typical hosts of *Pseudomonas syringae* pv. *syringae* such as lilac, bushbean, soybean, tomato, sugar beet, stone fruits, wheat, corn, sorghum, coffee and citrus were not infected by leaf infiltration of representative coriander strains.
A screening of different cultivated and wild growing species of the *Umbelliferae* showed that only few species reacted susceptible (Table 1). Most of the tested species reacted resistant or weakly resistant towards leaf infiltration and umbel spray inoculation.

Tab. 1: Symptoms on leaves and umbels of *Umbelliferae* species 14 days after inoculation with the coriander strain of *Pseudomonas syringae*

	Leaves					Umbels				
Inoculum dose ($cfu \times ml^{-1}$) Investigated plants	10^7	10^6	10^5	10^4	10^3	10^7	10^6	10^5	10^4	10^3
Coriandrum sativum	n	n	n	n	+	++	++	++	++	++
Levisticum sativum	n	n	n	+	+	++	+	+	+	+
Ammi majus						++	++	++	++	+
Heracleum sphondylium	n	n	n	-	-	(+)	(+)	-	-	-
Heracleum persicum	n	n	n	-	-	(+)	-	-	-	-
Bifora radians						(+)	-	-	-	-
Daucus carota	n	n	-	-	-	(+)	-	-	-	-
Carum carvi	n	n	-	-	-	-	-	-	-	-
Petroselinum sativum	n	n	n	n	-	(+)	-	-	-	-
Foeniculum vulgare						(+)	-	-	-	-
Pastinaca sativum	n	n	n	-	-	-	-	-	-	-
Apium graveolens	n	n	-	-	-	-	-	-	-	-
Anthriscus cerefolium	n	n	-	-	-	-	-	-	-	-
Anthriscus sylvestris						-	-	-	-	-
Anethum graveolens						(+)	-	-	-	-
Pimpinella anisum	n	-	-	-	-	(+)	-	-	-	-
Pimpinella major	n	n	-	-	-	-	-	-	-	-
Pimpinella saxifraga	n	n	-	-	-	-	-	-	-	-
Aethusa cynapium	n	n	-	-	-	(+)	-	-	-	-
Orlaya grandiflora	n	n	-	-	-	-	-	-	-	-
Aegopodium podagraria	n	n	-	-	-	-	-	-	-	-

n = necrosis - = no symptoms (+) = small non persistent water-soaked lesions
+ = persistent water-soaked lesions
++ = umbels covered with many water-soaked lesions

DISCUSSION

The studies indicate that umbel blight of coriander in West-Germany is caused by a hitherto undescribed *Pseudomonas syringae* pathovar. Similar results were obtained by Taylor and Dudley (1980) in Great Britain, Perez et al. (1990) in Mexico, and Cooksey et al. (1991) in California. The isolates of Taylor (e.g. NCPPB 3115) were, as far as investigated, very similar to the West German strains.

In contrast, Nemeth et al. (1969) described a fluorescent *Pseudomonas* species as the incitant of a coriander disease with similar symptoms in Hungary. Plescher (1983) investigated damaged coriander plants in East Germany and isolated a complex of different bacteria, including *Pseudomonas* spp., *Xanthomonas* spp. and *Erwinia* spp.. However, investigating more than 200 diseased plants from 15 different regions we always isolated large numbers of the described pathogen from plant tissue with typical symptoms. In a few cases low numbers of *Pseudomonas viridiflava* cells were identified additionally, but these were not capable of causing typical disease symptoms on coriander plants.

The host range studies showed that the described pathogen is a new pathovar of *Pseudomonas syringae*. The name *Pseudomonas syringae* pv. *coriandricola* is proposed.

One typical coriander isolate and one identical isolate obtained from wild carrot have been included in the National Collection of Plant Pathogenic Bacteria, Harpenden, U.K. (NCPPB nos. 3781 and 3780, respectively).

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Identification of plant pathogenic bacteria in broad bean (*Vicia faba* L.) seeds

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ABSTRACT

In broad bean seeds, wet necrotic spots were observed near the insertion of the funicle. Soon after germination, the resulting seedlings, showed a black wet rot covering the base of the stem and spreading upwards.

Phytopathogenic bacteria were isolated from small pieces of seeds as well as from the epicotyls showing already black spots.

On the basis of symptomatology, morphological, cultural and physiological characteristics, the bacteria studied were identified as *Erwinia carotovora* var. *atroseptica* (HELLMERS & DOWSON) DYE 1969, a phytopathogenic agent recorded for the first time in Portugal in broad beans.

KEYWORDS

Black wet rot, *Erwinia carotovora* var. *atroseptica*

INTRODUCTION

In the region of Anadia, in January, 1992, a disease was observed in young broad bean plants which showed dark necrosis on the stem base.

Using seeds from the stock from which the mentioned plants had been obtained, it was found that soon after germination, necrotic lesions causing soft root occurred near the funiculus (Fig. 1). Afterwards, in the plumule, dark lesions appeard which, in some cases, affected the whole tissue.

After the study of the symptoms, it was concluded that they were similar to the ones caused by phytopathogenic bacteria. Therefore, with the aim of clarifying the problem, isolation attemps of possible bacteria present in the affected material were done, for subsequent identification of the causal agent of the disease.

MATERIALS AND METHODS

Isolates

Isolates were obtained in potato dextrose agar (PDA) and nutrient agar with 5% sucrose (NSA), using small pieces of necrotic tissues. These pieces of tissue were first tranferred,after washing in sterilized destilled water, to a 2% sodium hypochloride solution. They were subsequently washed again in sterilized destilled water and macerated in a mortar. With the sap produced, Petri dishes were inoculated, using the method of streak, in order to obtain bacterial colonies. From these colonies, the bacterial cultures were obtained.

Pathogenicity tests

Pathogenicity was assessed by inoculating broad bean seedlings, obtained from healthy seeds, with a suspension containing 10^8 colony forming units (cfu) per mililiter, in sterilized destilled water. The inoculation was done by

hypodermic injection of the suspension in the stems. Alternatively, the tissues were pricked with a dissecation needle carrying inoculum removed from the Petri dishes containing solid medium with PDA or NSA.

After the inoculation, the plants were kept for 6 days in a wet chamber at temperatures near 20°C and then transferred to greenhouse benches.

Biochemical characterization of the cultures

Morphological characteristics

Gram staining: performed according to BARTHOLOMEW (1962).

Motility: observed under the optical microscope by the hanging-drop method on a microscope slide.

Flagella staining: the method of LEIFSON (1951) was followed.

Biochemical and physiological characteristics

Carbohidrate metabolism: determined by the method of HUGH & LEIFSON (1953).

Lipase production: assessed by the method of SIERRA (1957) using Tween 80 (polyoxyethylene sorbitan monooleate)

Acid production from sugar and related carbon compounds: media C and YS of DYE (1968) were used.

For all the other determinations the methods described by FERNANDES & PINTO-GANHÃO (1981) were followed.

RESULTS AND DISCUSSION

The bacterial cultures, obtained from tissues with soft rot near the epicotil or from necrotic tissued in the plumule, when inoculated in broad bean seedlings and young plants, produced pathogenicity in all cases.

The isolates obtained from the inoculated young plants showing necrosis in the base of the stems were also pathogenic.

From the plants infected in the pathogenicity tests, it was possible to re-isolate bacteria which were identical to the ones initially isolated.

The study of morphological, cultural and biochemical characteristics of the isolated cultures led to the conclusion that they consisted of rod-shaped, gram-negative bacteria with motility through several peritrichial flagella. It was also found that they do not produce spores and, in NSA medium, they form moderate and circular colonies. These colonies are cream-white, smooth, slihtly grayish, with a metallic luster, and a greenish iridescence in direct light.

The bacteria metabolize fermentatively glucose and hydrolize gelatin but not starch or Tween 80. They possess catalase and ß-glucosidase (in esculin medium). In addition, they are oxidase-negative, catalase-positive and do not origin fluorescent pigments in B-medium of KING et al.(1954). They also reduce nitrate and produce ammonia but not hydrogen sulphide or indol. They are positive in tobacco hypersensitivity test and potato soft rot (protopectinase activity), grow in the liquid medium with sodium chloride up to 5% and do not stand temperatures above 36°C in water bath.

Concerning the utilization of organic substances it was found that they grow with production of acidity on dextrose, arabinose, levulose, xylose, lactose, palatinose, maltose, rafinose, manitol, salicine, tartarate and α - methyl-glucoside.

Finally, it was found that they have a production of reducing substances from sucrose, and do not grow in media containning melezitose, i-inositol or starch. Oxidation of gluconate was not observed.

The symptoms in seeds and in the broad bean plants, together with the characteristics mentioned above, are identical to the ones described by several authors, such as ELLIOT (1951), DYE (1969), LELLIOT (1974), DICKEY (1979) and LELLIOT & DICKEY (1984) for *Erwinia carotovora var, atroseptica* (HELLMERS & DOWSON), DYE (1969).

This phytopathogenic bacterial species is here reported for the first time in Portugal on broad bean (*Vicia faba L.*) plants.

TABLE 1. Results of physiological and biochemical tests confirming the presence of Erwinia carotovora var. atroseptica, isolated from Vicia faba L.

TEST	RESULT
Aerobic (Hugh and Leifson test)	- (a)
Gelatin hydrolise	+
Starch "	-
Tween 80	-
Catalase reaction	+
ß-glucosidase activity (in esculin medium)	+
Oxidase	-
Fluorescence on King's B medium	-
Nitrate reduction	+
Production of ammonia	+
" " hydrogen sulphide	-
" " indol	-
Gluconate oxidation	-
Tobacco hypersensitivity	+
Potato soft rot (protopectinase activity)	+
Growth in 5% sodium chloride	+
Growth at 36°C	-
Reducing substance (sucrose)	+
Acid production from:	
Dextrose	+
Arabinose	+
Levulose	+
Xylose	+
Lactose	+
Palatinose	+
Maltose	+
Rafinose	+
Malezitose	-
Starch	-
Manitol	+
i-Inositol	-
Salicine	+
α-methyl-glucoside	+
Utilization of:	
Sodium malonate	-
" tartarate	-

Symbols: + all strains positive; - all strains negative;
(a) fermentative.

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Diagnostics of diseases in *Pseudomonas syringae*-infected plants

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ABSTRACT

With the purpose to indicate antigens from *Pseudomonas syringae* and diagnostics of bacterial diseases of plants, special preparation have been developed by express-method. The mechanism of preparation effect in based on the interaction of staphylococcal protein A and antibodies to antigen sought for. The optimum parameters for the treatment of staphylococcal reagent, its sorption and biological activities have been determined. Specificity and sensitiveness of the diagnostic preparations with respect to *Pseudomonas syringae* antigens have been studied. The possibility for indication of *Pseudomonas syringae* antigens in pure culture and infected plant material was demonstrated by express-method using diagnostic preparations proposed.

KEYWORDS

Pseudomonas syringae, bacterial antigens, bacterial diseases of plants, diagnostics, antisera, protein A.

Recently the bacterial diseases of plants became widely spread because of the intensification of agriculture, rising anthropogenic loading on nature and worsening ecological situation. In aetiology of plant diseases the bacteria of genus *Pseudomonas* causing the various types of injuries, i. e. bacterial spot, local lesions, tumour and rot, and as a result, the losses of yield are considerable. For prophylaxis and control of the diseases the discovery of the affected plants and correct identification of the agent are of great significance. The methods based on the interaction of bacterial antigens and specific antibodies are the most perspective.

With the purpose to find antigens of *Pseudomonas syringae* and diagonose bacterial diseases in plants by immunological express-method we have prepared specific preparations.

The rabbit immune sera for the detection of *Pseudomonas syringae* in the reaction of agglutination and double diffusion in agar have been used (PASTUSHENKO and SIMONOVITCH, 1979; YAKOVLEVA *et al.*, 1990), namely: sera to exocellular O-specific polysaccharide from *Pseudomonas wieringae* 7923 (YAKOVLEVA *et al.*, 1990); nine serogroup sera to the nine homologous strains of *Pseudomonas syringae* (PASTUSHENKO and SIMONOVITCH, 1979); polyvalent immune sera obtained by mechanical mixing in equal volume of the above serogroup sera. The titres of antisera have been determined in the reaction of agglutination in cells. Sera had the titres from I:25600 to I:52200.

Protein A-containing Cowan-I strain of *Staphylococcus aureus*, *Pseudomonas syringae* strains (II), bacteria of genus *Erwinia* (I), *Xanthomonas* (4), *Pseudomonas wieringae* (I), *Pseudomonas putida* (I), *Pseudomonas fluorescens* (2), *Pseudomonas seruginosa* (I) were received from the Collection of Department of Phytopathogenic Bacteria, Institute of Microbiology and Virology, Academy of Sciences of Ukraine, and Museum of cultures, Department of Microbiology and General Immunology, Kiev University.

Pseudomonas wieringae was classified as belonging to sero-group IV by PASTUSHENKO and SIMONOVITCH (1979).

To examine biological activity of staphylococcal protein A, specific diagnostic preparation was used which is glutar aldehyde-fixed and tannin-treated sheep's erythrocytes sensibilized by rabbit serum. The determination was carried out in the reaction of haemagglutination (PERADZE and HALONEN, 1985).

The control of sorption capacity of staphylococcal reagent in relation to rabbit immunoglobulins was performed by technique we had developed using method of Bradford's protein determination. Staphylococcal reagent was loaded with IgG of rabbit

serum, incubated at 37°C for one hour, and then staphylococci with adsorbed immunoglobulins were separated by centrifugation from supernatant which contained non-adsorbed immunoglobulins and other ballast substances. A suspension of native staphylococcal cells in 0.15 M solution of sodium chloride served as a control. The quantity of the protein adsorbed on staphylococcal reagent was calculated from the difference between the protein amount in serum used for sensibilization and its quantity in serum after sensibilization. Protein amount which could be washed from cell surface of staphylococcus into supernatant, was also taken into account. Sorption capacity was expressed in percentage as regards to protein concentration in serum.

Reaction of co-agglutination was carried out on slides, the calculation was performed according to 4-ball system visually with the unaided eye or under microscope (KRONVALL, 1973).

The different plant species grown in greenhouse of Institute of Microbiology and Viroly of Academy of Sciences Ukraine and in the fields of Kiev region have been used in experiments. For artificial infection of the plants, the various *Pseudomonas* strains have been selected.

The results obtained showed that optimum time for the cultivation of *Staphylococcus aureus* st. Cowan-1 on plane agar for obtaining protein A-containing corpuscles is 12-14 hours that coinsides with the data of other authors (HAZENSON, 1983).

Sorption capacity of the living staphylococcal cells as regards to immune serum is the highest in 12-14-hour agarized culture. Shortening cultivation period decreased both protein A content and biomass yield. Prolongation of the cultivation dropped the sorption capacity and biological activity of the preparation, possibly as a result of protein A washout under the effect of proteolytic enzymes. For the inactivation of staphylococcal suspension as well as for cell stabilization and the attachment to their surface of immunologically active protein A, additional treatments (heating and incubation with ethanol° were needed.

The heating at 80° C for one hour and treatment with 50% ethanol solution at 37°C for one hour were found to be optimum. The preparation activity after varied treatment was also checked in reaction of co-agglutination. For this purpose, staphylococcal preparation was treated with the correspondent immune serum. Sensibilization process was carried out for 30 min at 37°C with the subsequent wash-out from immunoglobulins not attached to protein A. Thus, at the use of formaldehyde-treated staphylococcus the positive reaction was observed at the concentration of antigens from 1.10^8 to 1.10^7 cell/ml. It was estimated as 2+, and accounting of the reaction could be done not earlier then 40-50 min

after beginning of the reaction. Reagent used with staphylococcus treated with ethanol solution determined the same antigen in the same concentration for 30 sec - 15 min, and the reaction was estimated as 4+. To check biological activity of protein A the reaction of indirect haemagglutination was used. The minimum dilution of staphylococcal suspension exibiting activity by protein A was 1.10^5 cells/ml (ethanol-treated), 1.10^9 cell/ml after formaldehyde treatment. In our tests this activity was not registered if only heating was used some days after storage.

Series of experiments on express-indication of *Pseudomonas syringae* agents and search for total antigens in the reaction of co-agglutination among representatives of nine serogroups of *Pseudomonas syringae* (PASTUSHENKO and SIMONOVITCH, 1979) have been carried out.

Diagnostic preparation used with antisera to some homologous strains appeared to be ineffective for the indication of the representatives of the species as a whole. Diagnostic preparations to serogroups II, V, VII, VIII were useful for the exposure of the antigens of the given serogroups since they possessed specificity to them. The other preparations gave cross reactions with antigens of several sero-groups, and, hence could not be used as the diagnostic preparations. The application of co-agglutinating reagent with antisera to exocellular 0-specific polysaccharide of *Pseudomonas wieringae* 7923 revealed *Pseudomonas syringae* antigens of serogroup IV (VIKHOT et al, 1991).

Use of polyvalent antiserum for obtaining diagnostic preprations makes it possible to determine the representatives of all nine serogroups in reaction of co-agglutination (CHEUSOVA et al, 1991). The data achieved from study of spectrum of action, specificity and sensitiveness of the diagnostic preparations have been used for agent indication in the infected plant material. Co-agglutinating reagent used with antiserum to exocellular 0-specific polysaccharide of *Pseudomonas wieringae* 7923 has revealed antigen in plants infected with *Pseudomonas syringae* strain of serogroup IV; the reagent used with polyvalent antiserum exposured the antigens of the members of all nine serogroups of *Pseudomonas syringae*

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Characterization of *Xanthomonas campestris* pathovars by rRNA gene restriction patterns

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Genomic DNA of 192 strains of the *Pseudomonadaceae* family, including 188 strains of the genus *Xanthomonas*, was cleaved by *Eco*RI endonuclease. After hybridization with 2-acetylamino-fluorene labelled (AAF) *Escherichia coli* 16+23S rRNA probe, 27 different patterns were obtained. Strains are clearly distinguishable at the genus, species, and pathovar level. Variability of rRNA gene restriction patterns was studied for four pathovars of *Xanthomonas campestris* species. The 16 strains analysed of *X. campestris* pv. *begoniae* gave only one pattern. For X. *campestris* pv. *manihotis* variability of rRNA gene restriction patterns could be related to ecotypes. On the contrary, the variability of patterns observed for *X. campestris* pv. *malvacearum* was neither correlated to pathogenicity nor to the geographical origins of the strains. The highest degree of variability of DNA fingerprints was observed within pathovar *dieffenbachiae*, which is pathogenic to several hosts of the *Araceae* family. In this case, variability could be related to both host plant and pathogenicity.

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Comparison of electrophoretic protein profiles within streptomycete strains causing common scab of potato in Québec

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In Québec, common scab of potato is mostly caused by two groups of streptomycetes, *Streptomyces scabies* and *Streptomyces* sp.. Most strains of S. *scabies* induced shallow lesions on potato tubers whereas isolates from *Streptomyces* sp. caused deep-pitted scab. Non-pathogenic strains phenotypically similar to *S. scabies* were also isolated from infected tubers. Analysis of electrophoretic patterns of soluble proteins on SDS-PAGE is a reliable method for studying the diversity among bacterial isolates. Proteins from *S. scabies*, from deep-pitted scab-inducing streptomycetes and from saprophyte strains phenotypically similar to S. scabies were extracted as follows. Cells were disrupted by sonication and the lysate was boiled in the presence of SDS (1%, final concentration). Thirthy µg of proteins were loaded on to the gel. Following electrophoresis, proteins were stained and electrophoretic patterns were analysed. High similarity was found within electrophoretic profiles of deep-pitted scab-inducing streptomycetes. Isolates of *S. scabies* and of non-pathogenic isolates phenotypically similar to *S. scabies* could be subdivided into two groups with a low degree of similarity. However, pathogenic and non-pathogenic strains were found in the two groups.

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Characterization of *Erwinia carotovora* strains with isozymes and restriction fragment length polymorphisms in relation to their pathogenicity on potato and chicory

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ABSTRACT

In order to provide new tools for epidemiological studies, detection and identification purposes, and to resolve the apparent taxonomic complexity of the subspecies, we investigated isozymes patterns and restriction fragment length polymorphisms (RFLP) of a natural collection of seventy strains of *Erwinia carotovora* isolated from different hosts and geographical areas.

Moreover, sixty five strains were inoculated on potato tubers and stems, chicory leaves and tobacco (H.R).

Pectinases, endoglucanases and proteases patterns from culture supernatants or cell lysates were investigated by isofocusing or polyacrylamide gel electrophoresis. RFLP were analysed using depolymerizing enzymes encoding probes: polygalacturonase, endoglucanase and pectine lyase (gifts from SAARILAHTI and CHATTERJEE); and ribosomal RNA (16S+23S).

Phenetic characters as well as RFLP could differentiate *Erwinia carotovora* ssp *atroseptica, ssp odorifera, ssp betavasculorum* and *ssp carotovora.* The phylogenetic tree revealed four main clusters: E.c.a, E.c.o and E.c.b groups appeared to be homogeneous groups, whereas E.c.c strains could be divided into at least four subgroups.

Pathogenicity on potato, chicory and tobacco could also separate the strains into four clusters strongly correlated to those obtained with phenetic and genetic characters.

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Improved taxonomy of the genus *Xanthomonas*

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In an extensive study of the relationships within the genus *Xanthomonas*, we have investigated over 900 strains belonging to all *Xanthomonas* species and *X. campestris* pathovars by means of SDS-PAGE of proteins and fatty acid analysis. Representative strains were further selected for genomic analysis by DNA-DNA hybridization. In addition to the species *X. albilineans, X. fragariae, X. maltophilia* and *X. populi*, which constitute separate DNA homology groups, the species *X. campestris* is heterogeneous and comprises at least 12 discrete DNA homology groups. Most of these groups consist of several pathovars that are unrelated in terms of phytopathogenic specialization. Others however, comprise pathovars from related hosts, e.g. the group of pathovars from grasses and cereals (*Poaceae*) and the group of pathovars from crucifers (*Brassicaceae*). The genus *Xanthomonas* needs to be reclassified in taxa that are acceptable from a general taxonomic point of view. Consequently, and in contrast to earlier classifications of *Xanthomonas*, the future species classification will not be correlated to phytopathogenic specialization.

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Taxonomy of some pathovars of *Pseudomonas syringae*

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ABSTRACT

In 1967, 29 species of phytopathogenic *Pseudomonas* were listed in group I and II according to the scheme of Lelliott et al (1966).
Presently 45 pathovars of *Pseudomonas syringae* are approved by the ISPP subcommittee on taxonomy of phytopathogenic bacteria. In addition about nine not valid pathovars of *P. syringae* are mentionned in the litterature.
The taxonomy of this group of phytopathogenic bacteria is very confuse. Moreover it is very difficult to identify each pathovar only by biochemical and physiological characters. It is the reason why we began to work on the taxonomy of some pathovars of *P. syringae* by numerical taxonomy and DNA/DNA hybridization.
By numerical taxonomy we demonstrated phenotypic variability of 108 strains of *P.s.* pv. *syringae* isolated from cherry laurel and many various host-plants. The strains were clustered in 7 groups which were differentiated by 26 biochemical characters. In comparison with pathovars of *P. syringae*, ninety three of 108 strains of pathovar *syringae* were clustered in one phenon with pathovars *atrofaciens, dysoxyli, apatata, panici, papulans* and *aceris*. This phenon was also differentiated by biochemical characters.
One hundred forty two strains of *P.s. savastanoi* isolated from six hosts (olive, oleander, ash, privet, phillyrea and jasmin) were clustered in one phenon which was differentiated by biochemical characters from the other pathovars of *P. syringae*.
Recently we demonstrated also that the following pathovars of *P. syringae* : *mors-prunorum, tomato, glycinea, porri, percicae* constituted separated phenons. *P.s.* pv. *pisi* was also clustered with the phenon pathovar *syringae* described formerly. We will study some other pathovars in order to find a determinative scheme.

By DNA/DNA hybridization we demonstrated that *P.s.*pv *savastanoi, P.s.* pv. *glycinea* and *P.s.* pv. *phaseolicola* constituted single DNA hybridization group and we have proposed to elevate P.s. pv. *savastanoi* at species level as *P. savastanoi.*

Recently we prooved that *P.s. pv. syringae, P.s.* pv. *dysoxyli, P.s.* pv. *aptata, P.s.* pv. *panici, P.s.* pv. *papulans, P.*s. pv. *aceris, P.s. lapsa* and *P.s.* pv. *pisi* constituted a single DNA hybridization group. We could be consider that these pathovars constitue a single species. It would be essential now to confirm the cross pathogenicity of these pathovars. Probably some may be synonyms. In a next step, to clarify the taxonomy of this group of phytopathogenic bacteria it will be essential to circumscribe DNA homology group among all these pathovars of *P. syringae.* I suggest to create an international working group on the taxonomy of *Pseudomonas syringae* pathovars.

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Use of bacteriophage for identification of *Xanthomonas campestris* pv. *translucens*

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ABSTRACT

Bacteriophages pathogenic to *Xanthomonas campestris* pv. *translucens* (Xct) were isolated from wheat (*Triticum aestivum L.*) growing in the field at Aberdeen, ID. Infected peduncle tissue (5g) was macerated in 15 ml of sterile, distilled water, centrifuged and filtered with a 0.45µ "µStar" (Costar Corp.) filter. The cleared supernatant (0.5 ml) was mixed with 1 ml of an overnight culture of Xct in 25 ml of 0.7 % nutrient agar (NA), and 3 ml of the mixture were poured over solidified 1.7 % NA. Plaques (lytic zones) developed in less than 24 hr at 25 C. Phage from single plaques were purified and stored in nutrient glucose broth. Thirty nine strains of Xct were screened against 47 phage isolates. Plaques were either clear on Xct strains pathogenic on wheat or turbid on strains pathogenic on barley. Three phage isolates were able to distinguish pathogenic strains of Xct from nonpathogenic strains recovered from storage at -80 C. Plaques were not observed with 23 strains of other pathovars and species of *Xanthomonas, Pseudomonas, Erwinia*, and *Clavibacter* tested against the three phage isolates. An additional 82 strains of Xct from Arkansas, Georgia, Mexico, and Brazil were also tested against the three phage isolates, and all reacted positively. Studies are planned to explore the relationship of phage virulence and pathogenicity of Xct.

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Use of serological affinities and phage sensitivity for the characterization of *Xanthomonas campestris* pv. *malvacearum* and its races

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ABSTRACT

Six phages isolated from cotton field soils of the Gezira -Sudan using strains of *X.c.malvacearum* as propagating strains were tested against four Xanthomonas species including fourteen *X.campestris* pathovars and over forty strains of *X.c.malvacearum* of the I.N.R.A. Versailles collection. Serological relations of the same isolates were studied using polyclonal antisera raised against whole cells of two Sudanese cotton pathotypes of *X.c.*pv.*malvacearum* using enzyme-linked immunosorbent assay (ELISA)

The phage sensitivity tests showed that isolates of *X.fragariae, X.axonopodis, X.albilineans,*and *X.campestris* except the pathovar *malvacearum* were insensitive to the phages. Strains of pv.*malvacearum* ,however, were very heterogenous in their phage reaction.Few were found to react with all the phage group thus being identical to race 2 of Sudan. This was further confirmed by pathogenicity tests (Freigoun et al.Introductry Session).Non-pathogenic isolates included within the pv.*malvacearum* collection were found to be insensitive to any of the phages and their morphological colony characteristics question their classification as *Xanthomonas.*

All the strains of *Xanthomonas campestris* pv.*malvacearum*; with the exception of the non-pathogenic isolates and pathovars of *X.campestris* tested reacted positively ,to varying degrees with the antisera using the APC method. This confirms the close serological relationship among the group and suggests the need for utilization of more specific antigenic determinants such as those associated with phage sensitivity,if any ,for raising polyclonal antibodies for characterization purposes. However when tested using DAS-ELISA only the pathovars *oryzicola, phaseoli* and *begoniae* and the species *albilineans* cross reacted with the antisera.

The results of this study emphasize the importance of using more than one parameter for distinguishing X.c.pathovars and their strains.

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Comparison of immunological methods for detection of *Erwinia carotovora* subsp. *atroseptica* using monoclonal and polyclonal antibodies

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ABSTRACT

Monoclonal antibodies (MCA) specific to *Erwinia carotovora* subsp. *atroseptica* (Eca) were raised against whole cells of the bacterium. One selected MCA was tested for speficificity in ELISA using 81 Eca strains, 117 *E.carotovora* subsp. *carotovora* (Ecc) and *E. chrysanthemi* strains and 100 strains of potato epiphytic flora. The MCA reacted only against all Eca strains assayed except 2 strains of De Boer serogroups 18 and 20 and 4 strains of unknown serogroup. Six antisera were produced against Eca but the polyclonal antibodies (PCA) were not specific to Eca in immunofluoresence nor ELISA.

The efficiency of different immunological techniques was compared using PCA, MCA or combinations of them to ensure specificity. The antigens used were pure culture of Eca and artificially inoculated and naturally infected potato tubers. A number of ELISA variants, indirect immunofluorescence, immunofluorescence colony plating and enzyme-linked immunoelectrotransfer (ELIET, Western blot and ELISA immunostaining), were studied comparatively.

The most sensitive methods were DAS-ELISA biotinstreptavidin system with previous enrichment of the samples in the plates, indirect immunofluroescence and immunofluorescence colony plating using the MCA. They were able to detect 100 cel of Eca/ml. ELIET using polyclonal antibodies was able to detect the same number of cells and to characterise specifically not only Eca but also Ecc in the potato samples.

These simple, accurate and reproducible immunological techniques are suitable for large scale testing of plant bacterial samples.

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Serological variability of isolates of *Pseudomonas corrugata* Roberts & Scarlett

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ABSTRACT

Pseudomonas corrugata, described in 1978 as the causal agent of tomato pith necrosis has been reported in many countries. However, there is not a comprehensive study of the phenotypical properties of this bacterium. Diversity of serological features of 128 isolates has been studied: 54 Spanish isolates from tomato, 2 Spanish isolates from pepper and 72 isolates from different countries were included. Three antisera were raised against type strain NCPPB 2445 and Spanish strains 536-71 from tomato and 592644 from pepper. Serological relationships (SR) were studied by indirect immunofluorescence and indirect ELISA. Each antisera displayed no cross reactivity with any of the two other strains. No antiserum was able to react against all the isolates. The antiserum against the type strain reacts in ELISA with 29.7% of the isolates, the antiserum from the tomato isolate reacted with 7.8% of them and the antiserum from the pepper isolate reacted with 17.2% of the isolates. When ELISA reaction occurred, the calculated SR was very variable. A high number of isolates (45.3%) did not react with the produced antisera. All these results show a high variability among antigenic determinants of *P. corrugata*. There was no correlation between the geographical origin or host of the isolates and SR.
The role of O-specific antigens of lipopolysaccharides (LPS) in the serological reactions has been studied by electrophoresis and immunobloting (enzyme-linked immunoelectrotransfer). Each antiserum reacted with whole cell lysates giving two common bands for *P. corrugata* isolates and other *Pseudomonas* and a ladder-like pattern characteristic of LPS. After proteinase K treatment the common bands dissapeared. Each antiserum reacted producing a different LPS pattern, indicating that *P. corrugata* seems to be also a heterogeneous species at the LPS level.

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Characterization of *Xanthomonas populi* races

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ABSTRACT

The aggressivity of *X. populi* strains was found to vary according to host genotypes, suggesting the occurrence of several physiological races within this bacterial species. This was verified by testing relative susceptibility of six poplar clones with 20 *X. populi* strains. Four races of *X.populi* strains were determined, which showed different degrees of aggressivity on two groups of poplar clones.

The *X. populi* strains belonging to different physiological races and different pathovars (*X.populi* pv. *populi* and *X.populi* pv. *salicis*) were analyzed by SDS-PAGE of proteins, by fatty acid profiles and at molecular level using the rrn IGS/RFLP fingerprint of intergenic spacer of the ribosomic operon (rrn IGS), and by sequencing the rrn IGS of four representative strains. There existed no correlation between *X.populi* physiological races and SDS-PAGE of proteins, fatty acid profiles as well as by sequencing. Although *X.populi* pv. *populi* and pv.*salicis* could not be differentiated from each other by SDS-PAGE of proteins or by fatty acid profiles, the rrn IGS sequence of pathovars *populi* and *salicis* were found to be different by two bases out of about 400 bp.

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Development of a DNA probe for detection of *Erwinia carotovora* subsp. *atroseptica*

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ABSTRACT

In Sweden, *Erwinia carotovora* subsp. *atroseptica* (*Eca*) causes blackleg of potatoes. The aim of the present work was to develop a DNA probe specific for Eca. The strategy used was the "shot-gun" method where randomly cloned DNA fragments are screened for specificity. A genomic library was obtained by cloning restriction fragments of EcoR1-digested DNA from one Eca isolate in the plasmid vector pUC19. Initially, 100 recombinant clones were investigated for their content of Eca inserts. The fragment size ranged from 300 bp to >5000 bp. A nonradioactive labelling and detection system, where digoxigenin is incorporated into the DNA, was used (Boehringer Mannheim). The specificity and sensitivity of the probes were investigated using dot blot DNA hybridization. Homologous DNA to the *Eca* probe could be detected down to pg level and total genomic DNA from Eca to ng level. Furthermore, dot blot with *Eca* cells, which were treated as for colony hybridization, was carried out and gave a positive reaction. However, the probes tested so far also gave a weak, positive reaction with DNA from *Erwinia carotovora* subsp. *carotovora* (*Ecc*) and *Ecc* cells. The screening for a specific DNA probe continues.

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Detection and identification of pectinolytic erwinias using PCR and RFLP

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ABSTRACT

Pectinolytic Erwinias : *Erwinia chrysanthemi* (Ech), *Erwinia carotovora* subsp. *atroseptica* (*Eca*) and subsp.*carotovora* (*Ecc*) responsible for various diseases on several plants including potato (wilt, soft rot, blackleg). Specific, rapid and sensitive detection tests are still required to prevent disease extension and to ensure plant quality.

PCR (Polymerase Chain Reaction) is such a sensitive technique which could be used for identification of pectinolytic erwinias at the level of the species, subspecies and possibly pathovars as well as for phylogenic studies or determination of the geographical origins.

Ech are mainly pathogen in warm climat or in glasshouses. Eca and Ecc are particularly pathogen under cold and temperate climat. Although those bacteria belong to the same species, mainly Eca is responsible of serious damages on potato (blackleg disease). It is then important to discriminate them.

For this purpose we retain genes involved in pathogenicity. After sequences analysis, two pairs of primers have been chosen. One set allows specific detection of *Ech* strains among a collection of prokaryotic or eukaryotic microorganisms, pectinolytic or not (see A. Nassar *et al.*) The second set recognizes specifically Ec strains. In order to distinguish *Eca* from *Ecc* strains, RFLP study has been undertaken on the amplified region in correlation with RFLP and pathogenicity studies (see S. Priou *et al.*) One enzyme is sufficient to distinguish *Eca* isolated from potato. According to a phylogenic analysis, RFLP results give 3 main groups: the first one is very homogeneous and contains all *Eca* isolated from potato, the second group is composed mainly by european and typical strains of *Ecc* and the members of a newly defined subspecies *odorifera*, and the third group by exotic or atypical *Ec* strains.

The real objectif of this work is to develop quick and reliable diagnosis kits for pectinolytic erwinias using the above results. Sensitivity studies of the *in vitro* test and on biological samples are underway. Based on results of the litterature, it is reasonable to expect the detection of ten to one bacteria in a sample. Such a detection level may provide a good tool for diagnosis and in addition for epidemiological studies.

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Studies of the host specificity of *Erwinia chrysanthemi* using PCR and RFLP methods

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Erwinia chrysanthemi is a pectinolytic enterobacterium responsible of soft rot and wilt diseases on various plants.

A specific PCR (Polymerase Chain Reaction) test has been developped using sequences limited to the ORFs of the pectate lyases (*pel*) A, D and E genes present in *E. chrysanthemi*. The *pel* genes are thought to be involved in pathogenicity.

The polymorphism of 83 *E. chrysanthemi* strains was studied by RFLP (Restriction Fragments Length Polymorphism) analysis on PCR amplified DNA fragments. The three restriction enzymes *AluI*, *HpaII* and *Sau3AI* provided 10 to 14 different patterns among the whole collection of strains.. The combination of patterns obtained with these enzymes revealed 20 RFLP groups.

A polymorphism was observed in pectate lyases (PL) isoenzymes separated by electrofocusing methods. The 24 PL profiles obtained matched the RFLP groups.

The phenetic analysis showed that the combined use of PCR/RFLP methods allowed the distribution of 83 *E. chrysanthemi* strains into 7 clusters. These clusters are correlated with the pathovars, the biovars and, to a lesser extent, to the geographical distribution.

The PCR/RFLP combined methods proved to be an accurate tool namely for taxonomic and epidemiologic purposes.

IV
Pathogenicity and host specificity determinants

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Molecular genetic analysis of pathogenicity and avirulence genes from *Xanthomonas campestris* pv. *vesicatoria*

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ABSTRACT

Xanthomonas campestris pv. *vesicatoria* causes bacterial spot disease in pepper and tomato. The *hrp* genes (hypersensitive reaction and pathogenicity) are organized in six loci clustered in a 25-kb region. *hrp* gene expression is induced in the plant and in plant cell culture filtrates. The *hrpF* locus can also be induced in a defined minimal medium without any plant-derived molecule. Sequence analysis revealed striking similarities between putative *Hrp* proteins and proteins from other bacteria, including the mammalian pathogens *Shigella* and *Yersinia*, that are essential for protein secretion. Based on these sequence similarities, we hypothesize that hrp gene products are involved in transport. Induction of the HR in resistant pepper and tomato plants requires *hrp* genes and a particular avirulence gene in *X.c. vesicatoria*. The avirulence genes *avrBs3* and *avrBs3-2* are highly homologous but differ in specificity. Both genes express repetitive proteins containing 17.5 copies of a nearly identical repeat motif. Sequence and genetic analyses of *avrBs3* alleles have demonstrated that the order of the repeats determines the specificity of the Avr-protein.

INTRODUCTION

Xanthomonas campestris pathovar (pv.) *vesicatoria* is the causal agent of bacterial spot disease of pepper and tomato plants. After infection of a plant with *X. c. vesicatoria* one of two different types of interactions can occur. If the interaction is compatible, infection gives rise to water-soaked lesions first which later on become necrotic. In contrast, in an incompatible interaction, i. e., when a resistant plant is infected, a hypersensitive response (HR) is induced. The HR is a local plant defense reaction accompanied by rapid necrosis of the infected plant tissue leading to an inhibition of bacterial growth in the intercellular space of the plant tissue. It has been shown that the incompatible interactions between *X. c. vesicatoria* and pepper are examples of gene-for-gene systems. This

means that a resistance gene in the plant is matched by a corresponding avirulence gene in the particular race of the pathogen (MINSAVAGE et al., 1990).
Using genetic and molecular approaches a number of bacterial genes involved in the interaction with the plant have been isolated. We are particularly interested in two types of genes, namely in *hrp* (hypersensitive response and pathogenicity) genes and in avirulence (*avr*) genes from *X. c. vesicatoria*. *hrp* genes that are essential for induction of the HR in the resistant plant and for development of disease in the susceptible plant and have been isolated from a number of different phytopathogenic bacteria (see review by WILLIS et al., 1991). To study the incompatible interaction between *X. c. vesicatoria* and resistant host plant cultivars we concentrate on the *avrBs3* avirulence gene family (BONAS et al., 1989).

RESULTS AND DISCUSSION

hrp genes from *X. c. vesicatoria*

Genetic analysis of the *hrp* region

Recently, a chromosomal region containing the *hrp* gene cluster from *X. c. vesicatoria* has been isolated by complementation of nonpathogenic NTG mutants (BONAS et al., 1991). Different cosmid clones, with overlapping inserts covering a genomic region of approximately 40 kb, were chosen for further analysis. After mutagenesis of these plasmids in *E. coli* using the transposon Tn*3-gus*, more than 60 different insertions were introduced into the genome of *X. c. vesicatoria* by marker gene exchange. Most insertions into a 25-kb region eliminated both pathogenicity and the ability to induce the HR on resistant pepper plants and on the nonhost tobacco. In addition, the nonpathogenic mutants have lost the ability to grow in the plant tissue. Complementation studies revealed that the *hrp* region of *X. c. vesicatoria* contains at least six complementation groups (loci), designated *hrpA*, *hrpB*, *hrpC*, *hrpD*, *hrpE*, and *hrpF*, the 4 kb region between *hrpE* and *hrpF* does not appear to be essential for the interaction with the plant because insertions in this region are phenotypically wild-type (BONAS et al., 1991; WENGELNIK & BONAS, unpublished data).

Expression of the *hrp* loci is regulated by the environment

To determine the conditions for expression of the *hrp* loci, we used the β-glucuronidase (*gus*) gene as a reporter gene. A promoterless *gus* gene is

present in the transposon Tn*3-gus* which was used for insertion mutagenesis of the cloned *hrp* region. The β-glucuronidase (GUS)-activity of *X. c. vesicatoria* strains harboring derivatives of the plasmids pXV2 and pXV9, carrying a Tn*3-gus* insertion in one of the *hrp* loci, was determined after bacterial growth in minimal or in complex medium; in no case was significant GUS-activity detected (SCHULTE & BONAS, 1992a). However, after growth of the bacteria in the plant, for each *hrp* locus at least one insertion with plant-inducible GUS-activity was found. In addition, culture filtrates from plant cell suspension cultures induce expression of all *hrp* loci. The culture filtrate from tomato suspension cultures, tomato conditioned medium (TCM), gave the highest activities, comparable to those obtained in pepper plants. MS, the basal medium for TCM, did not induce *hrp* expression. To isolate the putative plant factor(s) present in TCM, the properties of the inducing activity were determined: organic, heat-stable, hydrophilic, and smaller than 1000 Dalton (SCHULTE & BONAS, 1992a).

In order to mimic the conditions within the intercellular space of the plant tissue and to answer the question whether a plant factor is essential for *hrp* induction, we searched for a defined medium that would allow both growth of the bacteria and *hrp* gene induction. Since a *X. c. vesicatoria* merodiploid strain carrying pF312, with a Tn*3-gus* insertion in the *hrpF* locus, gives the highest inducible GUS-activity, this strain was used as a test strain. We designed the XVM1 medium, which supports bacterial growth and induces *hrpF* to high levels (SCHULTE & BONAS, 1992b). The XVM1 medium is a basal salt medium, low in both phosphate and sodium chloride concentration, and contains 10 mM sucrose and 2 μg/ml methionine as organic compounds. Interestingly, both sucrose (or fructose) and methionine are required for *hrpF* gene induction. High concentrations of phosphate, sodium chloride and organic nitrogen were found to suppress gene induction. The other *hrp* loci are less well or not induced under these conditions. It is not clear, whether additional factors are required to induce the other *hrp* loci or whether the XVM1 medium still contains components in suppressing concentration (SCHULTE & BONAS, 1992b).

What is the function of *hrp* genes?

As a first step towards answering this question, the nucleotide sequence of the entire *X. c. vesicatoria hrp* cluster will be determined. Recently, we have found striking sequence similarities to known proteins for some of the 12 putative Hrp proteins encoded by the *hrpA*, *hrpB*, and *hrpC* operons. The putative Hrp proteins HrpA1, HrpB3, and HrpC2, are similar to the YscC, YscJ, and LcrD proteins, respectively, from *Yersinia* species which are mammalian pathogenic

bacteria (FENSELAU et al., 1992). For example, HrpC2 shares 70% similarity to LcrD. In yersiniae, the causal agents of bubonic plague or gastroenteritis, the corresponding proteins are encoded by genes localized on a 70-kb virulence plasmid, and they are essential for secretion of important virulence factors, the so-called Yop proteins (CORNELIS et al., 1989). The putative *X. c. vesicatoria* HrpB6 protein is 65% similar to FlaA, FliI, and Spa47 proteins from *B. subtilis*, *S. typhimurium*, and *Shigella flexneri*, respectively. Interestingly, these related proteins are ATPases that are involved in protein transport, e. g., the Spa47 protein in *Shigella* is essential for the export of virulence proteins (FENSELAU et al., 1992). We hypothesize that one of the functions of Hrp proteins might be the transport of molecules that are involved in the interaction with the plant. These findings indicate for the first time that some of the basic pathogenicity mechanisms may be conserved among bacterial pathogens of animals and plants.

Analysis of the *avrBs3* gene family

Structure of *avrBs3* alleles

The avirulence gene *avrBs3* was isolated from a *X. c. vesicatoria* race 1 strain and is responsible for the induction of the HR on pepper cultivar ECW-30R which carries the *Bs3* resistance gene (BONAS et al., 1989). Southern blots of total genomic DNA of different strains of *X. c. vesicatoria*, probed with the internal 3.3-kb *Bam*HI fragment of the *avrBs3* gene, revealed in a number of strains the presence of a 26-kb *Eco*RI fragment (carries the *avrBs3* gene) and a 15-kb *Eco*RI fragment homologous to *avrBs3*. As described for *avrBs3*, the homologous sequence is also plasmid-borne. The 15 kb *Eco*RI fragment was isolated and found to contain an avirulence gene. In contrast to *avrBs3*, this gene induces an HR in tomato cultivars (e. g. Money Maker and Bonny Best) but not in pepper. Since the gene is highly homologous to *avrBs3*, it is considered to be allelic to *avrBs3* and, therefore, designated *avrBs3-2* (BONAS et al., 1992).

The DNA sequence of *avrBs3-2* revealed that the gene encodes a protein of 1160 amino acids which is 97% identical to AvrBs3. Interestingly, the internal regions of both *avrBs3-2* and *avrBs3* contain 17.5 copies of a nearly identical 102-bp repeat motif. Basically the same repeat motif is present in both genes, with a variable region at amino acid positions 12 and 13. The organization or order of the repeats, however, is different. This most likely is the reason for the different specificities of the two genes and is corroborated by the fact that new

avirulence specificities could be generated by deleting repeat units in the original *avrBs3* gene (HERBERS et al., 1992).
No sequence similarities between *avrBs3-2* and "unrelated" sequences in the databank were found. However, the 1.7 kb sequence of the *avrBsP* gene is 100% identical to the corresponding region in the *avrBs3-2* sequence. The *avrBsP* gene was isolated from a different strain of *Xcv* and also confers avirulence activity towards tomato but not pepper (CANTEROS et al., 1991). We believe that *avrBs3-2* and *avrBsP* might be identical genes. The *pthA* gene from *X. citri* appears also to belong to the family of *avrBs3*-related genes: it confers avirulence activity towards bean and cotton when introduced into the respective pathovars of *Xanthomonas*. In contrast to findings with *avrBs3*, mutations in *pthA* lead to reduction in virulence of *X. citri.* The N-terminal region of *pthA* is identical to the corresponding sequence of *avrBs3* and *avrBs3-2*, and it contains the same kind of basic repeat motif of 102 bp (SWARUP et al., 1992).

Expression and activity of *avrBs3-2*

Expression of the *avrBs3-2* gene is constitutive, as was described for the *avrBs3* gene (KNOOP et al., 1991). Surprisingly, derivatives of *avrBs3-2* with deletions into the C-terminal region remain active in inducing the HR on tomato. The shortest active derivative encodes a protein which consists of the N-terminal 288 amino acids plus 3.5 repeat units. In contrast, deletions into the C-terminus of the *avrBs3* gene abolish avirulence activity (BONAS et al., 1992). The role of *avrBs3-2* and the other members of the gene family in eliciting the HR remains elusive.

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Genes involved in pathogenicity of *Xanthomonas campestris* pv. *citri*, causal agent of "citrus canker"

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INTRODUCTION

Xanthomonas campestris pv. *citri* (Hasse) Dye causes canker (Asiatic canker) symptom on the leaves, stems and fruits of *Citrus* spp. To initiate our genetic study on the pathogenicity of this bacteria, we have isolated *Tn5* insertion mutants which lost pathogenicity. Here, we report on the characteristics of these mutants and on the cloning of some of the corresponding wild type fragments.

RESULTS

Isolation of non-pathogenic mutants by *Tn5* insertion.

The suicidal plasmid, pSUP1021 (SIMON *et al.*, 1983), was used for the generation of *Tn5*-insertion mutants of *X. c.* pv. *citri*. The suicidal nature of pSUP1021 in *X. c.* pv. *citri* was confirmed by observing the loss of the resistance to chloramphenicol (Cm), and the random nature of the insertions was confirmed by Southern hybridization using pRZ102 as the probe. Presumptive transconjugants were screened for the loss of their pathogenic response in the leaves of *Citrus natsudaidai* Hayata. Out of 1,600 transconjugants, ten prototrophic mutants, designated as F1-F10, were shown to have lost the ability to cause typical canker symptom 10 days after inoculation. Inoculations were performed through the stomata by pressing the lubber pieces pasted with each transconjugants from abaxial side of the leaves.The loss of pathogenicity was confirmed by the inoculation of bacterial suspensions into intracellular space.

Although F8 did not shown any symptom even one month after the inoculation, the other mutants started to show some yellowing around the inoculation sites (without development of canker symptom) about twenty days after the inoculation.

Characterization of non-pathogenic mutants.

Southern hybridization analyses for *Eco*RI- and *Smal*- digests of the total DNA from these mutants using pRZ102 as the probe indicated that all of them except F5 and F7 probably derived from single independent insertions. As Tn5 insertions were observed at identical *Eco*RI fragments in F5 and F7, they may be considered as identical mutants. Though *in vitro* growth rate of these mutants in YP medium were indistinguishable from the one of wild type, *in planta* growths of mutants F5, F7, F8 and F10 were severely affected. Especially, when 10^7 CFU of F8 was inoculated into the intracellular space of *Citrus natsudaidai* Hayata leaves, CFU rapidly declined and no viable cell was detected two days after the inoculation. Since the failure of *in planta* growth has been commonly reported in other *hrp* mutants (WILLIS *et al.*, 1991), it was thought that F5, F7, F8 and F10 may harbour mutagenized *hrp* genes (if any). To test this possibility, the response of tobacco suspension cells to wild type strain and to the mutants were studied. This was performed by measuring chemiluminescence, the raise of which has been observed as a typical early event of hypersensitive reaction in non-host plant cells to bacterial plant pathogens (KEPPLER *et al.*, 1989). As severe inhibition in the production of chemiluminescence was noted with these mutants when compared to wild type strain, they may be suspected to be *hrp*-like mutants.

Isolation of clone homologous to *hrp* cluster of *P. solanacearum*.

BOUCHER *et al.* had reported that a cosmid clone (pVir2) of the *hrp* cluster of *Pseudomonas solanacearum* hybridized not only with other strains of *P. solanacearum* but also with various *Xanthomonas* species (BOUCHER *et al.*, 1987). From the cosmid library of wild type *X. c.* pv. *citri*, the clones containing regions homologous to *hrp* cluster of *P. solanacearum* were searched by colony hybridization using the subclones of two different *Eco*RI fragments of pVir2 (*Eco*RI fragments ; no. 2 and 3) as the probes. Two clones were obtained for each probes, but did not shown any homology each other. When the cosmid clones containing the region homologous to *Eco*RI fragment no. 2 of pVir2 were used as the probe for Southern hybridization analyses of the F1-F10 mutants, shifts of about 5 kb were observed only in the cases of F4 and F9.

Therefore F4 and F9, among ten isolated mutants, may be mutagenized in the region homologous to *hrp* cluster of *P. solanacearum*. Thus, they also may be considered as *hrp*-like mutants. However, when the cosmid clone containing the region homologous to fragment no. 3 of pVir2 was used as the probe, no shift was observed in any mutants. Thus, none of the F1-F10 mutants seem to be mutagenized in this region.

Isolation of clones of other gene involved in pathogenicity.

Using *Tn5*-inserted *Eco*RI fragment of F8 as the probe for the colony hybridization, two cosmid clones (pXCF15-52 and pXCF16-33) which possess the *Tn5*-inserted fragment in F8, were obtained. When pXCF15-52 was used as the probe for Southern hybridization analyses for F1-F10 mutants, the shift of the wild type band was observed only in the case of F8.

DISCUSSION

Since we have not observed yet the typical hypersensitive reaction for wild type by infiltration into tobacco and other plants, it could not be definitely concluded that the isolated mutants are *hrp* mutants. However, since F4 and F9 were shown to contain the region homologous to *hrp* cluster of *P. solanacearum*, they may be suspected to possess *hrp*-like phenotype. It should be pointed out that their viabilities in *C. natsudaidai* were indistinguishable from the one of wild type. On the other hand, the mutants F5, F7, F8 and F10 showed the loss of viability *in planta* which is one of the common phenotypes among other *hrp* mutants. Based on presently available informations on the phenotypes of *hrp* mutants, they may be also *hrp*-like mutants. The other four mutants, F1, F2, F3 and F6, remained to be characterized further.

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Identification of an avirulence gene family in *Xanthomonas oryzae* pv. *oryzae*

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Abstract

Genomic DNA from strains of *Xanthomonas oryzae* pv. *oryzae*, the bacterial blight pathogen of rice, hybridized to the *avrBs3* of *X. campestris* pv. *vesicatoria* in a manner that was indicative of a multigene family. Two genes were identified with avirulence activity corresponding to resistance genes *Xa-10* and *Xa-7* of rice. Each cosmid containing the avirulence gene also carried a second apparent homolog to *avrBs3*. Avirulence activity was correlated with only one of the homlogs. Sequence analysis of *avrXa10* reveals the gene to be very similar to *avrBs3* with the exception of the repeat domain. The *avrXa10* has 15.5 102 bp repeats compared to the 17.5 repeats of *avrBs3*. Sequence variations within the repeat sequence are also apparent. The *avrXa7* gene has not been sequenced. However, based on restriction enzyme analysis the repeat region is predicted to have approximately 25.5 copies. The timing of hypersensitivity symptoms in the resistance response varies with each resistance gene. The timing difference was correlated with the specific *avr* gene. Experiments are in progress to determine the cellular location of the gene product in the bacterium and the portions of the repeat structure that are critical for avirulence activity.

Bacterial blight of rice is caused by *Xanthomonas oryzae* pv. *oryzae* (Ishiyama) Dye and is a major disease of rice throughout Asia (OU, 1985). Races of *X. oryzae* pv. *oryzae* have been defined using rice cultivars containing different resistance genes (MEW, 1987). Race-specific interactions are thought to follow the gene-for-gene model (FLOR, 1955) which predicts that incompatible interactions are the consequence of positive functions

encoded by avirulence genes in the pathogen and corresponding resistance genes in the host. Avirulence genes have been cloned from several different species and pathovars of *Xanthomonas* (BONAS *et al.*, 1989; CANTEROS *et al.*, 1991; DE FEYTER *et al.*, 1991; MINSAVAGE *et al.*, 1990; RONALD & STASKAWICZ, 1988; SWANSON *et al.*, 1988; SWARUP *et al.*, 1992). The gene *avrBs3* from *X. campestris* pv. *vesicatoria* was consider to be particularly interesting in regards to *X. oryzae* pv. *oryzae*. DNA sequences related to *avrBs3* were detected in seven additional pathovars of *X. campestris* (BONAS *et al.*, 1989) suggesting that related genes were to be found in other pathogenic *Xanthomonas* species and pathovars. In fact, homologs from *X. campestris* pv. *vesicatoria*, *X. campestris* pv. *malvacearum* and *X. campestris* pv. *citri* recently have been cloned and shown to have avirulence activity (CANTEROS *et al.*, 1991; SWARUP *et al.*, 1992). Previous reports did not include the examination of *X. oryzae* pv. *oryzae*. Therefore, we examined different races of *X. oryzae* pv. *oryzae* for the presence of *avrBs3*-like sequences.

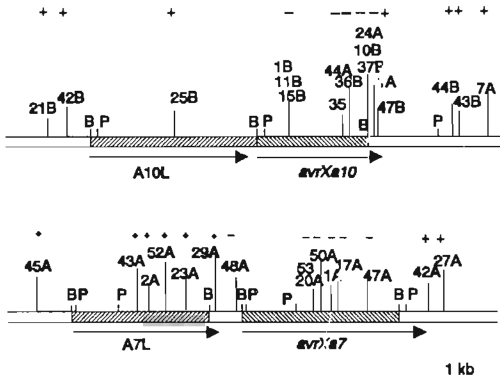

Fig. 1. Map of clones with *avrXa7* and *avrXa10* activity. B, *Bam*HI; P, *Pst*I. -, no avirulence activity; +, avirulence acitivty.

The presence of *avrBs3*-like sequences was determined by Southern hybrization under high stringency conditions (0.5 X SSC, 65°C) using the internal 3.3 kb *Bam*HI fragment from *avrBs3* (data not shown). (The *Bam*HI fragment was taken from pEC83, a full length clone of *avrBs3*, which was kindly supplied by Dr. B. Staskawicz.) All of the races of *X. oryzae* pv. *oryzae*

(from the Philippines) that were tested harbored multiple bands. Due to the complex patterns that were observed it is impossible to determine the exact numbers of genes represented in the hybridization pattern. Approximately 12 DNA fragments from race 2 strains ranging in size from 2.8 to greater than 12 kb were observed. Differences in banding patterns were observed in different strains. However, in general, the patterns among strains of the same race were similar. The result was consistent with the hypothesis that some of the apparent homologs might represent some of the avirulence genes that determine the race groupings.

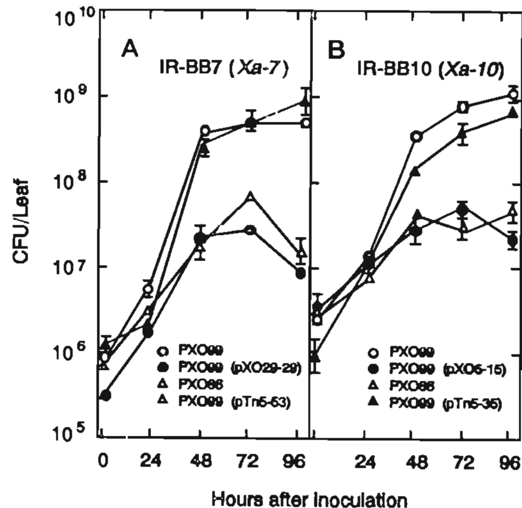

Fig. 2. Time course of bacterial growth in leaves of rice cultivars (A) IR-BB7 and (B) IR-BB10. Leaves were infiltrated with bacterial suspensions and were sampled for 96 h following infiltration. (A) IR-BB7 infiltrated with *X. oryzae* pv. *oryzae* PXO99 (○); PXO86 (△); PXO99(pXO29-29) (●); PXO99(pTn*5*-53) (▲). (B) IR-BB10 infiltrated with *X. oryzae* pv. *oryzae* PXO99 (○); PXO86 (△); PXO99(pXO5-15) (●); PXO99(pTn*5*-35) (▲).

To determine if any of the homologs of *avrBs3* from *X. oryzae* pv. *oryzae* were, in fact, avirulence genes, cosmids were selected form a genomic library of a race 2 strain (PXO86) on the basis of hybridization to the 3.3 kb fragment from *avrBs3* and tested for the ability to elicit resistance on a variety resistant rice cultivars. Forty-three clones were selected, and six clones, when mobilized into an otherwise virulent recipient (PXO99, race 6), conferred an avirulent phenotype upon inoculation of the appropriate cultivar. Two of the clones that appeared to harbor avirulence genes corresponding to the plant resistance genes *Xa-7* and *Xa-10*, respectively, were examined in more detail by restriction enzyme mapping,

transposon mutagenesis, and physiological analysis of the resistance reaction, i.e tissue browning, bacterial populations, and cationic peroxidase accumulation (REIMERS *et al.*, 1992). Both clones harbored two homologs of *avrBs3* (Fig. 1). Only mutations in the right copy (as arbitrarily drawn) correlated with the loss of avirulence activity. The bacterial populations were reduced in the inoculations of transconjugants on the appropriate cultivar, while the strains maintained virulence on a susceptible cultivar (Fig. 2). Peroxidase activity was also observed to increase in the inoculations with the transconjugants as was observed in the inoculations with the wild type bacteria

The presence of each avirulence gene also correlated closely with the timing of the resistance reaction on the respective resistant cultivar that was observed with the original race 2 strain. Plant reactions to inoculation by a race 2 strain involving *Xa-10* generally occur within 24 - 48 h, while reaction involving *Xa-7* appear to be slower requiring up to 72 h. The same timing was observed with the race 6 strain harboring *avrXa10* and *avrXa7*, respectively. The leveling off of bacterial populations and accumulation of inducible cationic peroxidase activity was delayed in the *avrXa7/Xa-7* combination compared to the *avrXa10/Xa-10* combination. These results are consistent with the hypothesis that the one avirulence gene and not a combination of genes is responsible for the reaction involving each resistance gene. It is possible that additional genes with the same avirulence activity are present in race 2 strains.

The *avrBs3* gene and the putative product is also interesting from a structural view point (BONAS *et al.*, 1989). The encoded protein is predicted to be 1163 aa in length. The approximate middle third consists of 17.5 tandem repeats of a 34 aa sequence. Recently, alterations in the repeat region of the gene were shown to alter avirulence activity and specificity (HERBERS *et al.*, 1992). The *avrXa10* from *X. oryzae* pv. *oryzae* was sequenced and found to be closely related to *avrBs3* by nucleotide and amino acid sequence identity. The *avrXa10* sequence is two repeats shorter than

avrBs3 (15.5 copies). Each nucleotide repeat is 102 bp in length representing 34 aa. Most of the sequence differences between the repeats of *avrBs3* and between the individual repeats of *avrXa10* occurred in a six base pair region hereafter referred to as the variable region. The variable region represents codons 12 and 13 of the repeat sequence (Fig. 3). Although some repeats are identical, there was no discernable order to the arrangement of repeats. Repeats of *avrXa7* and the leftward homologs that were linked to *avrXa7* and *avrXa10* were 25.5, 17.5, and 19.5 copies, respectively (data not shown). A third apparent avirulence gene was cloned corresponding to the *xa-5* resistance gene, and further analysis of the *avrXa5* gene is in progress.

```
                 VAR
1   LTPDQVVAIASNIGGNQALETVQRLLPVLCQAHG
2   LTPDQVVAIASHGGGKQALETVQRLLPVLCQAHG
3   LTPDQVVASASNIGGKQALATVQRLLPVLCQDHG
4   LTPDQVVAIASHGGGKQALETVQRLLPVLCQDHG
5   LTPDQVVAIASNIGGKQALETVQRLLPVLCQDHG
6   LTPDQVVAIASNIGGKQALETVQRLLPVLCQDHG
7   LTPDQVVAIANNNGGKQALETVQRLLPVLCQTHG
8   LTPDQVVAIASHDGGKQALETVQRLLPVLCQDHG
9   LTPDQVVAIASNIGGKQALATVQRLLPVLCQAHG
10  LTPDQVVAIASHDGGKQALETVQRLLPVVCQDHG
11  LTPDQVVAIANNNGGKQALETVQRLLPVLCQDHG
12  LTPAQVVAIASHGGGKQALETVQRLLPVLCQDHG
13  LTPVQVVAIASNSGGKQALETVQRLLPVLCQDHG
14  LTPVQVVAIASNGGGKQALATVQRLLPVLCQDHG
15  LTPVQVVAIASHDGGKQALETVQRLLPVLCQDHG
16  LTPDQVVAIASNGGGKQAL

        *                          *
1'  LTPEQVVAIASHDGGKQALETVQRLLPVLCQAHG
aa  1        10        20        30
```

Fig. 3. Amino acid sequence of the *avrXa10* repeat domain. VAR, variable region (shaded amino acids). Variant amino acids that are outside of the variable region are boxed. Repeat number is indicated at the left. Repeat 1' represents repeat of *avrBs3* (6). "*" indicates sequence variations of *avrBs3* compared to major residue in *avrXa10* repeat at the corresponding position.

This work extends the growing list of *avrBs3*-like genes that function to confer avirulence in dicotyledonous plant/ phytobacteria interactions to a monocot system. The sequence relatedness of the genes; the broad range of pathogens in which the genes occur; and the presence of multiple copies of the genes in several species leads one to suspect that the gene products play important roles in the physiology of the bacteria. The relationships also may reflect a similarity in the mechanism of host recognition and the elicitation in resistance among the broad range of host plants. Many questions remain regarding the mechanism by which the *avrBs3*-like genes function in the elicitation of race specific resistance.

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Host recognition of bacterial *avr* and *hrp* genes results in active oxygen production

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Studies of the early stages of plant/bacteria interactions using cell suspensions have indicated that active oxygen production increases during two phases. The first phase appears to be a nonspecific response occurring immediately after addition of the bacteria to plant cells. The second phase of active oxygen production occurs only with incompatible bacteria which cause a hypersensitive response. The bacterial genes responsible for plant recognition in two different systems involving either non-host recognition (*Pseudomonas syringae* pv. *syringae* and tobacco) or race-cultivar recognition (*P. s.* pv. *glycinea* and soybean) are necessary for stimulating both the subsequent hypersensitive response in leaves and the second phase active oxygen production in suspension cells. In *P. s.* pv. *syringae*, 12 of 13 complementation groups in the *hrp* cluster are necessary for both the hypersensitive response and active oxygen production; mutations in any of these groups block both responses. In addition, transfer of this *hrp* cluster to *P. fluorescens*, a non-pathogenic saprophyte, enables the bacterium to trigger both the hypersensitive response and the active oxygen response in tobacco. Transfer of the *avrA* gene of *P. s.* pv. *glycinea* from an incompatible race to a compatible race allowed recognition by the soybean cultivar Mandarin resulting in second phase active oxygen production as well as the hypersensitive response. Second phase active oxygen production appears to be a recognition response by the plant upon contact with bacteria containing incompatible *avr* or *hrp* genes.

Keywords: hypersensitive response, active oxygen, host recognition, hydrogen peroxide

Recognition of external stimuli, especially those emanating from pathogenic microbes, is essential for the survival of plants in nature.

Successful recognition turns on various inherent defense mechanisms and results in a resistant interaction. Recent evidence by our laboratory suggests that active oxygen metabolism is turned on during the recognition processes of plant/bacteria interactions. We have focused on incompatible interactions which lead to a hypersensitive reaction in leaf tissue.

Active oxygen. The active oxygen species most often involved in biological reactions result from the reduction of molecular oxygen. The one-electron reduction of ground state oxygen results in the production of the superoxide radical. Three subsequent one-electron reductions respectively yield hydrogen peroxide, the hydroxyl radical and, finally, water. The latter reactions occur spontaneously or in the presence of a reaction partner where no extra energy is required. Therefore, these reactants are termed active oxygen species. All of these active oxygen species are involved in numerous reactions, several of which allow them to interconvert. Therefore in biological inteactions, it is important to realize that the product being measured may not be the original product of the activated mechanism.

Hydrogen peroxide is a relatively stable form of active oxygen. At physiological pH any superoxide present is converted to hydrogen peroxide relatively fast. Therefore the amount of hydrogen peroxide present is a relative measure of the level of active oxygen in the system. To detect hydrogen peroxide we use a chemiluminescent assay in which luminol is oxidized to aminophthalate and emits a photon. The major advantages of this assay are that it allows detection of very low levels of active oxygen, it is rapid, and it is relatively nonintrusive to the system (Glazener *et al.*, 1991b).

Previous work. In previous studies we compared the active oxygen production of tobacco suspension cells incubated with compatible, incompatible and saprophytic bacteria (Baker *et al.*, 1991). This study allowed us to generate working hypotheses in regard to early responses associated with plant/bacteria interactions (Figure 1). 1) All bacteria stimulate a rapid increase in active oxygen levels (phase I) upon contact with plant cells. 2) Pathogenic bacterial interactions have a rapid decrease in active oxygen within 1 hr. 3) Saprophytic interactions are slow in decreasing active oxygen levels. 4) Incompatible (HR) bacterial interactions stimulate a burst of active oxygen (phase II) after about 2 hr.

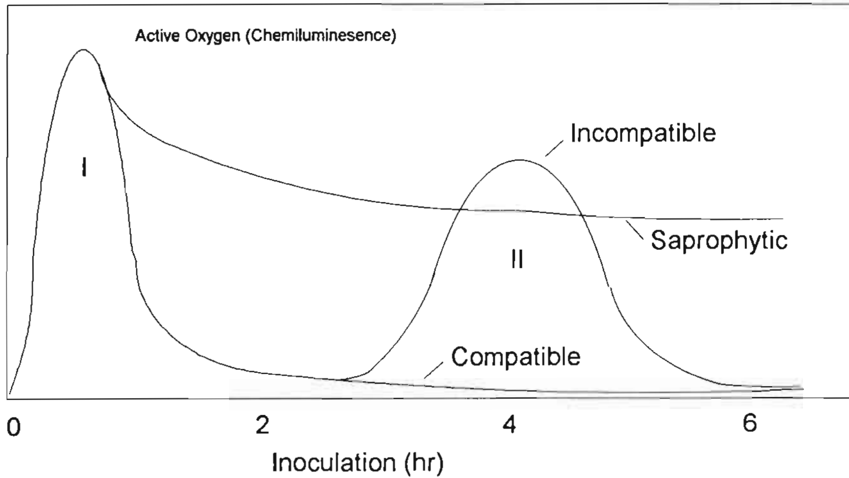

Figure 1. Diagram summarizing the active oxygen levels during the initial interaction between plant suspension cells and bacteria.

Effect of hrp genes. Recent studies using Tn*phoA* mutants of *P. syringae* pv. *syringae* confirm the association between incompatibility and phase II active oxygen production. Previously Huang *et al.* (1988) demonstrated that only a relatively small region of the *P. syringae* genome is required for the elicitation of the HR or K^+/H^+ response (net K+ efflux /H+ uptake). Acquisition of a plasmid containing this region (pHIR11) into *P. fluorescens* conferred the ability to cause HR on tobacco as well as to stimulate the K^+/H^+ response. Recently Glazener *et al.* (1991a) found that this same mutant was now able to stimulate active oxygen production during the second phase (Figure 2).

Huang *et al.* (1991) have found this region to consist of 13 complementation groups. Mutations in any of 12 of these groups blocked HR and the K^+/H^+ response. However, mutations in the complementation group I caused a delayed HR and a reduction in the K^+/H^+ response. Glazener *et al.* (1991a) tested these mutants and found similar results. Mutations in complementation groups 2 through 13 blocked phase II active oxygen production; mutations in complementation group I did not affect phase II. Therefore, it appears that in *P. s.* pv. *syringae* the entire *hrp* cluster, except for group I, is required for stimulating the second phase of active oxygen production in the plant.

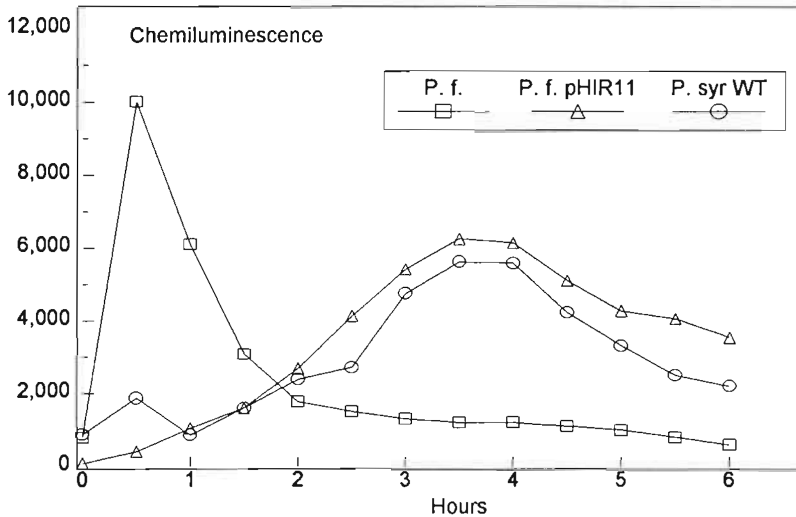

Figure 2. Active oxygen production by tobacco cells treated with *P. fluorescens* containing the pHIR11 region from *P. s.* pv. *syringae*. (P. f. = *P. fluorescens*; P. f. pHIR11 = transconjugant containing pHIR11; P. syr. WT = *P. s.* pv. *syringae* wild type strain)

Effect of avr genes. Studies similar to those described above for non-host recognition in tobacco were carried out for race-cultivar recognition in soybean. *P. s.* pv. *glycinea* race 4 (Psgr4) induced a compatible reaction on soybean cultivar Mandarin leaves while race 6 (Psgr6) induced an incompatible hypersensitive response (Glazener *et al.*, 1991a; Orlandi *et al.*, 1992). With Mandarin suspension cells, as expected, race 6 caused the phase II active oxygen response about 2.5 hr after inoculation (Figure 3).

Orlandi *et al.* (1992) recently reported that incorporation of a single avirulence gene, *avrA*, into Psgr4 caused the resultant Psgr4(pAVRA) mutant to induce not only a HR on Mandarin leaves but also the K^+/H^+ response in suspension cells and leaf disks and the phase II active oxygen response in suspension cells (Figure 3). Control mutant strains containing only the vector plasmid (pDSK519) without the *avrA* gene did not induce this response. This study provides strong support for association of the K^+/H^+ and active oxygen responses and the recognition of incompatible bacterial pathogens containing *avr* genes.

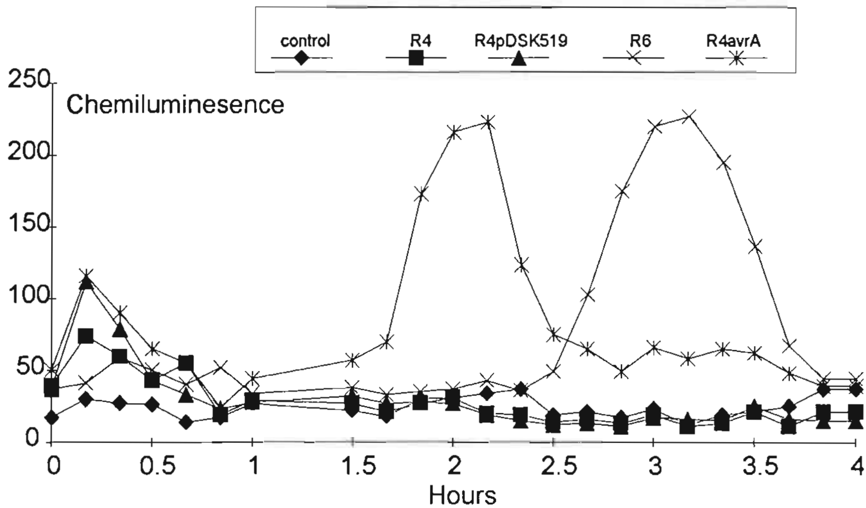

Figure 3. Active oxygen as monitored by luminol-dependent-chemiluminescence in soybean suspension cells treated with *P. s.* pv. *glycinea* races and transconjugants. (R4=*P. s.* pv. *glycinea* Race 4; R4(pDSK519)=*P. s.* pv. *glycinea* Race 4 transconjugant containing the plasmid pDSK519; R6=*P. s.* pv. *glycinea* Race 6; R4avrA=*P. s.* pv. *glycinea* Race 4 transconjugant containing pAVRA from Race 6.)

Conclusions. It appears that active oxygen production occurs during two phases of the early plant/bacteria interaction. Phase I occurs immediately after addition of bacteria and is non-specific; phase II occurs 2 - 3 hr later and is specific for HR-causing bacteria.

It is feasible that phase I production of active oxygen creates an oxidative stress in the surrounding tissue, similar to oxidative stress which occurs during senescence. Tissues exposed to increased levels of active oxygen undergo numerous changes and the physiological state could be altered to effect the plant's response to subsequent stimuli coming from the pathogen during phase II.

The phase II active oxygen increase seems to be stimulated as a result of the presence of *AVR* or *hrp* genes in the bacteria. Our previous studies indicate that the levels of superoxide are greater during this phase of active oxygen production. We have preliminary evidence indicating membrane-bound oxidases might be activated. Theoretically this activation would be through specific receptors, but at this point the data are inconclusive.

While it remains to be determined if active oxygen plays a key role in recognition, it seems clear that its production is a plant response to recognition of bacteria carrying specific *avr* or *hrp* genes. The transient nature of the reactants and products and the complexities of the metabolic pathways make it difficult to study active oxygen in plant systems. For this reason the role of active oxygen metabolism in many processes has previously gone unnoticed. Because of the tremendous potential impact of active oxygen species it is important that we be aware of its existence and strive to more fully understand its role in plant/pathogen interactions.

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Multiple regulations control *pel* gene expression in *Erwinia chrysanthemi*

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Abstract

Synthesis of pectate lyases, the main determinant of *Erwinia chrysanthemi* pathogenicity, is controlled by many regulatory genes. *kdgR* is the main regulatory gene, controlling all the genes of the pectinolysis. Others, such as *pecS*, act only on the expression of the *pel* genes. Some genes such as *gpi* and *cri* are global regulators. Finally, expression of each *pel* gene is modulated by specific genes such as *pecX*, *pecY* and *pecZ*.

Keywords: *Erwinia chrysanthemi,* pectate lyase, regulation.

Introduction

Synthesis and secretion of cell wall degrading enzymes is the main determinant of the pathogenicity of *E. chrysanthemi* (Collmer and Keen, 1986). Tissue maceration results from the action of pectin degrading enzymes such as pectin methylesterase and pectate lyases. The virulence of *E. chrysanthemi* depends on parameters such as temperature, humidity, oxygene pressure or iron concentration (Pérombelon, 1990; Expert and Gill, 1992). Moreover, synthesis of pectin degrading enzymes is inducible in the presence of pectin degradation products. Thus, synthesis of these enzymes seems to be under the control of complex regulatory mechanisms.

The genes coding for pectin degrading enzymes are distributed into two clusters on the bacterial chromosome: one contains the genes *pelD*, *pelE* and *pelA* coding for the pectate lyases PelD, PelE and PelA and the gene *pem* coding for the pectin methylesterase (Reverchon *et al.*, 1986). The other one contains the

genes *pelB* and *pelC* coding for the pectate lyases PelB and PelC. All these genes constitute independant transcriptional units. In order to better understand the regulation of the expression of these genes, several mutations affecting the expression of one or all of these genes have been isolated and the corresponding genes characterized. Some of these mutations affect the expression of the five *pel* genes (*kdgR*, *pecS*, *gpi*, *cri*). Other ones act specifically on the expression of one or two of these genes (*pecX*, *pecY*, *pecZ*).

Results

1- Genes involved in the regulation of the five *pel* genes

The gene *kdgR*

Previous physiological studies have shown that the expression of all the genes involved in pectin degradation (*pelA*, *B*, *C*, *D*, *E*, *pem*, *ogl*, *kduI*, *kduD*, *kdgK*, *kdgA* and *kdgT*) is induced in the presence of pectin or galacturonate. This suggests the existence of a common regulatory mechanism. Analysis of *pel* gene induction in various mutants showed that 2-keto-3-deoxygluconate (KDG), the common intermediate to the pectin and galacturonate degradation pathways, is the true inducer formed from these compounds (Condemine *et al.*, 1986, Hugouvieux-Cotte-Pattat and Robert-Baudouy,1987).

Mutants expressing constitutively one of the genes of the pectin degradation pathway were found easily, either spontaneously or after transposon mutagenesis. In these mutants the five *pel* genes and all the genes of the pectin degradation pathway are expressed constitutively. The mutated locus has been named *kdgR* (Condemine *et al.*, 1987). While the genes for the intracellular part of the pathway (*ogl* to *kdgA*) are expressed at their maximal level in a *kdgR* mutant, the *pel* gene expression is still inducible in presence of polygalacturonate. Thus, *kdgR* is probably not the only regulatory gene that controls *pel* gene expression. The expression of some of the genes involved in pectate lyase and cellulase secretion (*outT* and the *outC-O* operon) is also constitutive in a *kdgR* mutant, demonstrating that the *kdgR* regulation is not limited to the pectin catabolic pathway (Condemine *et al.*, in preparation).

The *kdgR* gene is located in a region of the chromosome that contains other genes involved in pectin catabolism (*ogl*, *kduD*, *kduI*, *kdgC* and *kdgF*). It has been cloned and sequenced (Reverchon *et al.*, 1991). It codes for a protein of a molecular weight of 35 029 Da containing 306 amino acids. The KdgR protein has

significant similarities to two prokaryotic regulatory proteins: the IclR protein involved in acetate degradation in *E. coli* and *S. typhimurium*, and the GylR protein involved in glycerol metabolism in *Streptomyces coelicolor*. A region that could form a helix-turn-helix motif and serve as a DNA binding region was detected from amino acids 77 to 96 (Reverchon *et al.*, 1991).

A conserved sequence of 27 bp has been found in the regulatory region of all the genes regulated by *kdgR* (Condemine and Robert-Baudouy, 1991). This sequence is almost palindromic, a characteristic of operator sequences: A(A/T)AAAA(A/T)GAAA(C/T)NNTGTTTCATT(A/T)T(A/T)T. KdgR is able to bind to this sequence *in vivo* and *in vitro*. Gel retardation experiments demonstrated that two KdgR dimers bind to an operator (Nasser *et al.*, 1992).

The role of KDG as a true intracellular inducer was confirmed by gel retardation assays: addition of KDG dissociates the KdgR-operator complex. The inducing properties of KDG analogs were tested using the same assay. All the inducing molecules contain the motif COOH-CO-CH2-CHOH-C-C included in a pyranic cycle (Nasser *et al.*, 1991). Thus, 5-O-Methyl KDG that cannot be metabolized, is a gratuitous inducer of the expression of all the genes regulated by *kdgR* (Nasser *et al.*, 1991).

The gene *pecS*

Other mutations, located in a different region of the chomosome, led to a constitutive expression of the five *pel* genes. The mutated gene was named *pecS* (Reverchon *et al.*, 1990). In *pecS* mutants, the level of expression of the genes of the intracellular part of the pathway is unchanged. Inactivation of *pecS* by insertion leads to a constitutive expression of the genes it controls. This strongly suggests that *pecS* is a negative regulatory gene. Regulation by *pecS* does not seem to involve any variation in the level of expression of *kdgR*. *pecS* has recently been cloned and sequenced. PecS is a leucine rich hydrophobic protein and could be a membrane protein. It has no significant homology to any known protein. The mechanism by which it regulates *pel* gene expression is still unknown.

The *gpi* and *cri* genes

Two types of mutations (*gpi*, *cri*) that affect *pel* expression (probably through changes in global regulation networks) have been found by NTG mutagenesis of the strain B374 (Hugouvieux-Cotte-Pattat *et al.*, 1986). In *gpi* mutants, the synthesis of PL and cellulase became independent of the growth phase, while in

the wild-type strain it strongly increases at the end of the exponential growth phase. *gpi* and *kdgR* mutations are located near the same locus and could be two different alleles of the same gene. In *cri* mutants, the expression of many genes become insensitive to the catabolite repression exerted by glucose. In such a mutant, PL synthesis increased about three-fold in induced or uninduced conditions. The *cri* mutations seems to act at the level of catabolite repression.

2- Specific regulatory genes

- The gene *pecX*

Insertion mutagenesis of the region upstream of *pelA* led to the identification of a gene regulating *pelA* expression (Favey, 1991). This gene, named *pecX*, codes for a protein with homology to globins and reductases. *pelA* is the only gene with a reduced expression in a *pecX* mutant in aerobic condition. However, in a *pecX* mutant grown in anaerobic conditions, expression of all the *pel* genes seems reduced. This gene could be involved in an oxygen-dependent regulation.

- The gene *pecY*

pecY is a short gene located between the *pem* and the *pelD* genes (Boccara *et al.*, 1989). An insertion in this gene leads to a constitutive expression of the *pem* gene. Thus, it could be a negative regulator of *pem* expression. A mutation in *pecY* does not affect the expression of the *pel* genes.

- The gene *pecZ*

Several evidences support the existence of a negative regulatory gene controlling *pelB* and *pelC* expression. Yankovski *et al.* (1989) and Hugouvieux-Cotte-Pattat and Robert-Baudouy (1992) found that the introduction of a plasmid containing the region downstream of *pelC* in a strain containing *pelB* and *pelC* reduced the expression of these two genes. No retardation of *pelB* or *pelC* operator regions could be observed by gel retardation with an extract of *E. coli* containing the cloned *pecZ* gene. Thus, the action of this gene could be indirect.

Conclusion

Regulation of the expression of the *pel* genes appears very complex and probably occurs at several levels. *pel* gene expression is modified by

environmental conditions through global regulatory networks. Some of the genes involved in these regulations have been identified. Amongst those, *gpi*, *cri* or *cbr* could be involved in regulation in response to variation in growth phase, catabolite repression or iron concentration (Expert and Gill, 1992) respectively. Other regulatory systems responding to temperature, osmolarity and nitrogen starvation have been observed at the physiological level using gene fusions (Hugouvieux-Cotte-Pattat, in preparation). Regulation by *kdgR* seems to be essential since it turns on the expression of all the genes involved in pectin degradation (pectate lyase genes, pectin catabolism genes and secretion genes). Finally specific regulatory genes such as *pecY* and *pecZ* modulate the level of each *pel* gene in response to unknown signals. Thus, *E. chrysanthemi* is able to adapt pectate lyase production to any condition.

Résumé

La synthèse des pectate lyases, le principal déterminant du pouvoir pathogène d'*Erwinia chrysanthemi* est controlée par de nombreux gènes régulateurs. *kdgR* est le gène régulateur principal, contrôlant tous les gènes de la pectinolyse. *pecS* n'agit que sur les gènes *pel*. Les gènes *gpi* et *cri* sont des régulateurs globaux. Enfin, l'expression de chaque gène *pel* est modulée par des gènes spécifiques tels que *pecY* et *pecZ*.

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Role of *Erwinia chrysanthemi* pathogenicity determinants in host specificity

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Abstract. Several pathogenicity determinants were characterized in the past decade, in *Erwinia chrysanthemi*, the causal agent of soft rot disease on many plant species. To assess the importance of these determinants in relation to the host, the pathogenic behavior of mutants affected in these various pathogenicity factors was compared in four plant systems, two isolated organs - potato tubers and etiolated chicory leaves - and two *in vitro* grown plantlets - pea and saintpaulia. This study brings strong evidence for an involvement of pectic enzymes in host specificity. It also allows us to show that Ech 3937 produces another set of pectinases, the production of which is induced only *in planta*.

The enterobacterium *Erwinia chrysanthemi* (*Ech*) is the causal agent of soft rot disease on many plant species in the field as well as under storage. One of the most extensively analyzed traits of this bacterium was its ability to produce and secrete a set of depolymerizing enzymes (pectinases, cellulases, proteases) which are able to degrade the constituents of plant cell walls. Only the pectinases were shown to play a crucial role in pathogenicity (for reviews see Collmer and Keen, 1986 ; Kotoujansky, 1987 ;Collmer *et al*, 1991). *Ech* strain 3937 produces five pectate lyases encoded by the *pelA* to *pelE* genes and one pectin methylesterase encoded by the *pem* gene. In this strain, several approaches have led to the identification of other determinants involved in the full expression of virulence. These include components of the bacterial envelope, e.g. lipopolysaccharide (LPS) (Schoonejans et al, 1988) and one of the major outer membrane proteins (OMP) (Beaulieu and Van Gijsegem,1992); a functional iron assimilation system (Enard et

al, 1988) as well as products of several bacterial genes inducible by plant extracts (Beaulieu and Van Gijsegem, 1990).

To assess the importance of these determinants in relation to the host, the pathogenic behavior of mutants affected in these various pathogenicity factors was compared in four plant systems, two isolated organs - potato tubers and etiolated chicory leaves - and two *in vitro* grown plantlets - pea and saintpaulia (Beaulieu and Van Gijsegem, 1992 ; Beaulieu *et al*, submitted). A summary of these pathogenicity tests are presented in Table 1. This allowed us to draw the following conclusions :

1) Potato tubers were found to be especially sensitive to *Ech* infection. Most of the *Ech* mutants tested retained their ability to macerate potato tubers as efficiently as the wild type strain. Only those mutants impaired in the production or secretion of multiple PL isoenzymes (PelADE$^-$, ΔPel and Out$^-$) and the Pem$^-$ mutant displayed reduced virulence in this system. However, this lack of discrimination is not due only to the fact that a tuber is an isolated organ since, in etiolated chicory leaves, virulence levels depended on the inoculated mutant.

2) Mutants affected in cell envelope (Omp$^-$, Lps$^-$), most Pin$^-$ (for Plant INducible) mutants and the PelA$^-$ mutant were reduced in virulence in the three other hosts. These determinants seemed thus to be involved in quite general mechanisms of virulence which are required irrespective of the plant infected. Amongst the Pel$^-$ mutants, only the PelA$^-$ one exhibited reduced virulence on all three plants. The *pelA* gene seems thus to be the only *pel* gene involved in general mechanisms of virulence. It is worth to note that, contrarily to the other isoenzymes, the production of the Pla isoenzyme is low in minimal media supplemented with polygalacturonate (PGA, i.e. demethylated pectin) representing only a few % of the total PL activity produced in this conditions (Reverchon *et al*, 1986)). The *pelA* gene expression is furthermore barely induced by the addition of PGA and it was shown that the purified PLa protein has no macerating ability on plant tissue (Barras *et al*, 1987). It was also recently shown that the degradation products produced by the action of the PLa isoenzyme on polygalacturonate are different to the oligouronides produced by the action of the other PL isoenzymes (Preston III *et al*, 1992). This may point to a role of Pla in the production of some signals more than a catabolic role in the destruction of plant tissue integrity. The importance of oligosaccharide signals both in plant defense responses and in plant development is indeed increasingly evident (Ryan and Farmer, 1991).

Table 1. Ratio of virulence efficiency between *Ech* mutants and the wild type strain 3937

Strain	Phenotype	Ratio of virulence efficiency on			
		potato tuber (a)	Saintpaulia (b)	chicory leaf (c)	pea (b)
PMV4066	PelA	0.84	0.41(d) *	0.85 *	0.47 *
PMV4070	PelD	0.84	0.47 (d) *	0.77 *	1.07
PMV4071	PelE	1.53 *	0.19 (d) *	1.22 *	0.73
PMV4120	PelBC	0.97	1.10 *	0.71 *	0.87
PMV4121	PelADE	0.61 *	0.32 *	0.67 *	0.53 *
PMV4116	Δ(Pel)	0.72 *	<0.03 *	0.69 *	0.41 *
PMV4103	Pem	0.74 *	0.11 (e) *	0.91	0.77
RH7001	Pin3	1.10 (f)	0.34 (f)*	0.24 (f)*	0.30 (f)*
RH7002	Pin6	0.80 (f)	0.55 (f)*	0.52 (f)*	0.36 (f)*
RH7003	Pin8	1.01 (f)	0.14 (f)*	0.20 (f)*	0.30 (f)*
RH7004	Pin9	0.95 (f)	0.21 (f)*	0.26 (f)*	0.30 (f)*
RH7005	Pin10	0.88 (f)	0.29 (f)*	0.28 (f)*	0.42 (f)*
RH7006	Pin14	1.05 (f)	0.29 (f)*	0.24 (f)*	0.76 (f)
RH7007	Pin23	1.07 (f)	0.83 (f)	1.04 (f)	0.84 (f)
RH7008	Pin29	0.96 (f)	0.55 (f)*	0.54 (f)*	0.54 (f)*
RH7009	Pin32	1.05 (f)	0.55 (f)*	0.66 (f)*	0.90(f)
RH7010	Pin38	0.83 (f)	0.98 (f)	1.21 (f)*	1.45 (f)*
RH7011	Omp	0.77 (f)	0.26 (f)*	0.30 (f)*	0.42 (f)*
RH7012	Rgr	0.93 (f)	<0.03 (f)*	0.88 (f)	0.18 (f)*
RH7015	Out	0.13 (f)*	<0.03 (f)*	0.12 (f)*	0.12 (f)*
R1456	Lps	0.77	0.07*	0.13*	0.47*

a) Ratio of the average weight of tissue rotted by a mutant of the wild type strain
b) Ratio of the number of plants presenting systemic symptoms when inoculated with a mutant and the wild type strain
c) Ratio of the average maceration speed on chicory leaves inoculated with a mutant and the wild type strain
d) Data from Boccara et al, 1988
e) Data from Boccara and Chatain, 1989
f) Data from Beaulieu and Van Gijsegem, 1992
* indicates that the virulence efficiency of the mutant is significatively different from that of the wild type strain ($p < 0.05$).

3) Expression of the other *pel* genes, most *pin* genes and the *rgr* (for Reduced GRowth) gene were needed for full expression of virulence only on one or two plants which strongly suggests that they might be involved in host specificity. Hence, *Ech* may possess specific mechanisms for dealing with the environmental conditions encountered in different plants. Quite surprisingly however, most of the Pin⁻ mutants which were thought to be involved in early mechanisms in the recognition between the pathogen and its host are not involved in such specificity. Rather, the pectinases seem to play a role in that respect, as the most striking differences in behavior on the different plants were exhibited by the Pel⁻ mutants. Comparisons of the effects of PelD⁻ and PelE⁻ mutations are especially interesting since the sequences of their respective structural genes show high homology and since these genes, when present, are conserved in the different strains of *Ech* (Van Gijsegem, 1989). Despite this homology, insertion mutations in the *pelD* and *pelE* genes led to different alterations in virulence. Whereas an insertion in *pelD* had no effect on potato tuber or a negative effect on chicory leaf maceration, an insertion in *pelE* resulted in increased virulence on these isolated organs. The upstream non-coding sequences of the *pelD* and *pelE* genes are divergent (Van Gijsegem,1989), so it is possible that the different behaviors of the Pel-defective mutants result from sequential, differential or interdependent expression of *pel* genes within the different plant species. This hypothesis was confirmed by the analysis of the PL content of fluids harvested from plants at late phases of infection (Beaulieu *et al*, submitted). The predominant PLs in macerated tissues were found to differ between plant species. Little correlation however was found between the preponderance of a particular enzyme at late disease stages and its importance in symptom development. It is clear that kinetic studies of the expression of the different *pel* genes *in planta* should be undertaken to analyze the role of these isoenzymes in the establishment of the bacterium-host interaction.

4) The maceration capability of a mutant deleted for the five *pel* genes is still unexpectedly efficient. Analysis by IEF of fluids from rotted plants inoculated with this mutant allowed the detection of a new set of pectinases, only expressed *in planta*. The IEF PL activity profiles found in fluids of different plants were not identical suggesting that, for this new set of pectinases also, there might be a differential production depending upon the infected host (Beaulieu *et al*, submitted). These newly identified pectinases are likely to be exported from the bacterial cells by the same secretion mechanism as the other PL enzymes, since an Out⁻ phenotype was associated with absence of symptoms in potato tubers (Beaulieu and Van Gijsegem, 1992). This was not due to an absence of cellulase secretion

since mutants impaired in the synthesis of cellulases are not impaired in their ability to macerate tubers (Aymeric *et al*, 1989).
Interestingly also, the ΔPel mutant shows unique pathogenic behavior on saintpaulia plantlets: all the infected leaves were at least partially rotted but the disease never passed to other leaves of the infected plant. After inoculation with the wild type strain on the contrary, sometimes, no visible symptoms were observed at the inoculation site. It is tempting to speculate that in the case of the ΔPel mutant, the ecognition of the pathogen by the plant is not as efficient anymore; this may allow some rotting through the action of the *in planta* induced pectinases but the sole production of these enzymes may not be sufficient to permit the invasion of a whole plant by the bacteria.

In conclusion, this study shows that the role of the different pectinolytic enzymes of *Ech* in symptom production on plants is host-dependent and suggests an involvement of these enzymes in host specificity. Other factors of course, must also contribute to determine host specificity. Molecular recognition between particular interacting organisms, adaptation of the pathogen to specialized environments and chemical composition of the plant may contribute to the determination of the particular host range of an *Ech* strain. Whatsoever, this study provides further insight into the significance of pectinase multiciplicity in *Ech*. Efficient maceration of the host by this set of enzymes clearly brings an advantage to the bacterium in providing nutrients derived from plant debris. Moreover, this multiplicity of enzymes, whose production may be tuned by the specific host, may reflect the evolutionary response of the bacterium to the enormous diversity of plant species.

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Studies on hairy root T-DNA: regulation and properties of ORF13 from *Agrobacterium rhizogenes* 8196

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Abstract: The possible role of ORF13 has been investigated through studies on transgenic tobacco plants carrying various gene constructs derived from ORF13. Transgenic plants in which ORF13 is expressed under the control of the 35SRNA promoter have an abnormal phenotype, with reduced growth, short internodes, leaf and flower deformation. This phenotype is not restricted to the scion but also observed on the normal stock on which a transgenic shoot has been grafted. This indicates that the expresion of ORF13 is responsible for the production of a morphogenetic substance which is translocated through the graft union to the stock whose development is consequently altered. Physiological studies on transgenic plants expressing ORF13 suggest that this gene acts by modulating cytokinin activity.
Regulation of expression of ORF13 has been studied using the ß-glucuronidase (GUS) reporter gene. GUS expression was found to be wound-inducible in a non-systemic fashion. Deletions in the 5' non-coding sequence upstream to the gene have been created in order to identify promoter regions involved in this regulation.

Keywords : *Agrobacterium rhizogenes* / ß-glucuronidase / cytokinins / mannopine / ORF13 / rhizogenesis / wound

INTRODUCTION

Agrobacterium rhizogenes induces on dicotyledonous plant species the hairy root disease. The molecular basis of this disease is the transfer and stable integration of a segment (the T-DNA) from a large bacterial Ri (for Root-inducing) plasmid into the plant genome (ZAMBRYSKI *et al.*, 1989; BIROT *et al.*, 1987). Even though the T-DNA of agropine strains of *A.rhizogenes* carries auxin synthesis genes, it has been shown that these are not essential for hairy root proliferation (SPENA *et al.*, 1987). In contrast to the T-DNA of *A. rhizogenes* agropine strains, the T-DNA of the mannopine strains 8196 does not carry any hormone synthesis gene. It has been shown that expression of T-DNA genes from this strain influences the hormone response by increasing auxin sensivity of transformed cells and organs (SHEN *et al.*,1990).

Three genes have been shown to be essential in agropine strains for hairy root proliferation; there are *rolA*, *B* and *C* or ORF10, 11 and 12 respectively (HANSEN *et al.*, 1991a; BREVET *et al.*, 1988; SPENA *et al.*,

1987). However 2 genes of the agropine Ri plasmid, the ORF 13 and 14 are necessary for hairy root induction on carrot discs (CAPONE *et al.*,1989). A striking point is that these genes are more conserved in different Ri plasmid-types (*ca* 77 % for ORF13) and less (*ca* 65 %) for *rolA, B, C* (HANSEN *et al*, 1991a). This suggest an important function for this genes.

1 EFFECTS OF ORF13 OVEREXPRESSION IN TRANSGENIC TOBACCO PLANTS

1.1 In order to study the biological effects of ORF13, a chimeric gene which contains the coding region of ORF 13 under the control of the 35S-promoter from the Cauliflower Mosaic Virus was constructed (HANSEN, 1991b). Transgenic tobacco plants which contain the construct were made. Shoots of these transgenic plants grow very slowly and exhibit altered morphology: the root system is reduced, the internodal distance is small and irregular, the leaves are wrinkled and dark green. Flowering is retarded but seed production is not reduced, the corolla of some flowers exhibit a sort of spur. In the selfed progeny, some plants show a more accentuated phenotype; some of their flowers have petaloid stamens.

1.2 35S-ORF13 transgenic shoots were grafted onto the cut stem of normal tobacco plants. The phenotype of the developing grafted shoots was typical of 35S-ORF13 plants although they showed better growth. But the striking point is that abnormal offshoots developped from the stock: they exhibited the ORF13 phenotype with small internodal distance, wrinkled and dark green leaves. When cuttings of these offshoots were made, the new growth reverted to the normal phenotype. When the reciprocal graft was made, the grafted shoot exhibited the 35S-ORF13 phenotype.

Thus expression of ORF13 influences plant morphology *via* a diffusible product. To our knowledge, this is the only case where the rootstock phenotype is influenced by the overexpression of an *A.rhizogenes* T-DNA gene.

1.3 Physiological comparison of the transgenic plants and normal plants showed that the overexpression of ORF13 results in impaired rooting capacity. Leaf discs from normal plants cultured *in vitro* on MS medium containing 5.10^{-6} M NAA rooted profusely. In contrast, discs from 35S-ORF13 leaves showed much reduced rooting capacity for every NAA concentration tested. Adding a very small amount of cytokinin (3.10^{-8} M Benzylaminopurine) nearly completely restored the rooting capacity of transgenic leaf discs. The same treatment did not modify the rooting capacity of normal leaf discs.

However 35S-ORF13 leaves are more green than normal leaves. They contain 1,5 times more chlorophyll than the normal leaves. This fact can mean that there is more cytokinin in 35S-ORF13 leaves than in those of normal plant.

These physiological studies therefore lead to opposite conclusions which, we believe, reflects a profound change in cytokinin responses of 35S-ORF13 plants.

2 REGULATION OF ORF13 EXPRESSION

2.1 To study the regulation of the ORF13 expression, the Gus coding sequence was placed under the control of ORF13 promoter. Transgenic plants were made (HANSEN, 1991b). Experiences showed that ORF13 is expressed at higher levels in roots than in leaves.

Moreover, histological staining showed that ORF13 was highly expressed in tissues at the site of lateral root emergence and in the region of the root hypocotyl junction, suggesting that tissue damages incurred during normal development activate ORF13 promoter. To test this hypothesis, we measure promoter activity after mechanical wounding.

2.2 ORF13 promoter was found to be wound-inducible in almost all tissues studied. Gus activity was increased, 24h. after wounding, about 70 fold in transformed leaf discs and about 15 fold in transformed roots

The kinetics of wound-induced response were investigated in transgenic tobacco plants containing the chimeric gene. 2 mm leaf discs were incubated in liquid medium. Gus expression started to increase 5 h after wounding to reach 70 fold stimulation after 22 h.

2.3 Histochemical staining showed that the induction by wounding of ORF13 Gus Gene is not systemic. When we wounded mechanically a leaf and cut off a fragment containing the wound 24h later, only cells close to the wound-site were stained.

ORF13 follows a rather complex pattern of expression, being under both developmental control (it is root-specific) and stress (it is wound-induced). This emphasizes the need to define the components of ORF13 promoter region which lead to developmentally controlled or wound induced activation.

3 STUDY OF PROGRESSIVE DELETIONS OF ORF13 PROMOTER

Deletions in the promoter sequence were made in order to find which element is responsible for the wound-induction. A series of deletions distributed over the entire promoter region was obtained. We studied the expression of Gus gene under the control of the different promoter deletions in carrot hairy roots.

a. The deletions were made on a *E.coli-Agrobacterium* shuttle plasmid. Carrot discs were inoculated with *A.rhizogenes* strains containing pRi8196 and, on the shuttle plasmid, the Gus gene under control of the deleted promoter. With such a binary transformation system about half of the hairy roots formed should contain the T-DNA carried by the shuttle vector in addition to 8196 T-DNA (PETIT *et al*, 1986).

b. Three weeks after inoculation, roots emerged from the carrot cambium. They were transferred onto culture media. As soon as the roots were long enough, they were used for Gus Activity measurements. 2 cm long fragments were cut in 1 or 2 mm slices and incubated in liquid medium. Gus activity was measured after 24 hours.

4 deletions distributed over the promoter region were analysed. The results of this experiment showed that induction by wounding was retained only for one of the deletions. This experiment allows us to conclude that the sequence responsible for wound induction must be located between 600 bp and 360 bp. upstream of the initiation codon.

A larger set of deletions should allow us to more precisely locate the sequence essential for wound-induction.

CONCLUSION

Several questions are raised by the work on ORF13
- What is the nature and the mode of action of the morphogenetic substance produced in 35S-ORF13 tobacco plants?
- What are the sequences and the factors involved in wound-induction of ORF13?
- Do other environmental stresses influence the expression of ORF13?
- What is the role of ORF13 in hairy root disease?

Wound-induction of the ORF13 gene suggests that this gene may play an important role in the first stage of hairy root induction.

Résumé :L'étude de la fonction de l'ORF13 a été abordée par l'étude de plantes contenant l'ORF13 sous le contrôle du promoteur 35S du CaMV. Ces tabacs transgéniques 35S-ORF13 montrent un ralentissement de croissance et des altérations morphologiques des feuilles et des fleurs. Lorsque ces tabacs sont greffés sur un tabac normal, le phénotype 35S-ORF13 est également observé sur les rejets du porte-greffe. La surexpression de l'ORF13 est donc responsable de la production d'un composé morphogène diffusible à travers l'union et influençant le développement du porte-greffe. Des études physiologiques des tabacs 35S-ORF13 montrent que l'ORF13 affecte le métabolisme des cytokinines.
L'étude de tabacs transgéniques contenant le gène reporter de la ß-glucuronidase (GUS) sous le contrôle du promoteur de l'ORF13 a permis de mettre en évidence une induction localisée importante du gène par la blessure. Des délétions dans la région 5' non codante du gène ont été réalisées pour déterminer les séquences impliquées dans cette induction.

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Do phenols induce *vir* genes of *Agrobacterium*?

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ABSTRACT

Pathogenicity of *Agrobacterium* depends on the induction of virulence genes. The induction of *vir* locus is attributed to phenols, sugars, pH and temperature. The primary event is the activation of cellular glycosidase that leads to the release of aglycones. The phenolic aglycone induces the *vir* locus.

KEYWORDS

Agrobacterium, *vir* gene, phenols.

INTRODUCTION

Agrobacterium tumefaciens causes crown gall, the neoplastic disease of most dicot plants. Tumor results from the transfer of a segment of Ti plasmid, designated as T-DNA (transferred DNA) which gets stably integrated into the plant nuclear genome. The T-DNA encodes enzymes for biosynthesis of auxin and cytokinin leading to hypertrophic condition and opines, novel sugar and amino acid conjugates (BINNS & THOMASHOW, 1988).

T-DNA packaging is a complex process initiated on activation of the *vir* regulon, comprised of seven complementation groups (*vir*A, *vir*B, *vir*C, *vir*D, *virE*, *vir*F and *vir*G) (STACHEL & NESTER, 1986a). The coordinate regulation of the *vir* genes is under the regulation of *Vir*A/*Vir*G two component regulatory system (WINANS *et al.*, 1988). The putative sensor molecule, *Vir*A is an inner membrane protein, which on perception of the environmental stimuli gets phosphorylated at a histidine residue in the cytoplasmic domain (HUANG *et al.*, 1990a ; JIN *et al.*, 1990a). Signal transduction proceeds via phosphorylation of *Vir*G activator protein at an aspartic acid residue by the autophosphorylated *Vir*A (JIN *et al.*, 1990b). Activated *Vir*G increases transcription of its own gene and induces the *vir* box, a dodecameric *cis*-acting regulatory sequence (TNCAATTGAAAY) in the promoter region of the inducible genes (WINANS *et al.*, 1988).

In addition to the Ti plasmid virulence genes, chromosome-encoded *chv*A, *chv*B, *exo*C (*psc*A) (CANGELOSI *et al.*, 1987 ; DOUGLAS *et al.*, 1985), *chv*D (WINANS *et al.*, 1988), *chv*E (HUANG *et al.*, 1990b) and *ros* (CLOSE *et al.*, 1985) loci are involved in crown gall tumorigenesis.

FACTORS INDUCING VIRULENCE GENES IN *AGROBACTERIUM*

In plant-microbe interaction, the primary event is recognition which involves signal perception, transduction and activation of the relevant bacterial genes leading to symbiotic or pathogenic interaction. Communication is through phenols present in plants, released upon wounding and infection by pathogens. Similarly flavonoids induce the nodulation genes in *Rhizobium* (ROSSEN *et al.*, 1987).

Agrobacterium virulence genes are induced by plant phenols like acetosyringone, -hydroxy acetosyringone (STACHEL *et al.*, 1985), monosaccharides and derivatives (ANKENBAUER & NESTER, 1990 ; CANGELOSI *et al.*, 1990 ; SHIMODA *et al.*, 1990) by acidic conditions (STACHEL *et al.*, 1986c ; WINANS *et al.*, 1988), inorganic phosphate starvation (WINANS *et al.*, 1988) and temperature (ALT-MOERBE *et al.*, 1988 ; RIKER, 1924). *Vir*G is transcribed from two promoters, one is constitutively expressed in the free-living bacteria and the other is induced in a *vir*A-*vir*G dependent manner (STACHEL & ZAMBRYSKI, 1986b). The degree of induction depends on the concentration of the inducing substance, but both promoters differ for various compounds due to variations in structure.

CHEMOTAXIS

Agrobacterium possesses a sensitive chemotaxis system responsive to a variety of sugars, amino acids and phenolics (LOAKE *et al.*, 1988). Chemotactic responses are largely encoded on chromosome (LOAKE *et al.*, 1988 ; PARKE *et al.*, 1987). However, chemotaxis toward acetosyringone requires the presence of Ti plasmid. *Vir*A and *vir*G trigger chemotaxis at low concentrations and *vir* induction at high concentrations (SHAW *et al.*, 1988). *Agrobacterium* infects the wound sites were there is an accumulation of phenolics.

The locus *chv*E mediates chemotaxis, uptake, and *vir* gene induction in response to galactose/glucose and several other sugars. It codes for a 31.5 kDa protein homologous to periplasmic ribose- and galactose/glucose-binding protein of *E. coli* (HUANG *et al.*, 1990b). A *chv*E protein, GBP1 was obtained from *A. radiobacter* (CORNISH *et al.*. 1989). The *vir*A is structurally similar to the Trg protein of *E. coli* which interacts with the ligand-bound GBP to transmit the chemotactic signal (PARK & HAZELBAUER, 1986).

For optimum Ti-plasmid determined chemotaxis and *vir* induction the presence of a 4'-hydroxyl group, 3' and 5' O-methyl groups, and a polar side chain at the 1' position appears essential (ASHBY *et al.*, 1988).

CATOBOLISM OF PHENOLICS AND INDUCTION OF VIRULENCE GENES

Phenols that mediate plant-pathogen interaction were found to induce catabolic genes in the latter. This phenomenon is exemplified by *Rhizobium* and *Agrobacterium* which exhibit many similarities in their respective nodulation and transformation processes (PETERS & VERMA, 1990). Enzymes like dioxygenase, demethylase and deacetylase, involved in catabolic pathways, may be correlated to the conversion of *vir* inducers of *Agrobacterium* through their degradation. The catabolic pathways are induced by specific substrates of pathways. One such *beta* -ketoadipate pathway is inducible in *A. tumefaciens*, *R. fredii*, *R. meliloti*, *R. leguminosarum* bv. *viciae* and *R. leguminosarum* bv. *trifolii* and constitutive in *Bradyrhizobium* (PARKE & ORNSTON, 1986). B-Ketoadipate pathway enables the utilization of monocyclic compounds, such as acetosyringone, sinapinic acid, coniferyl alcohol, syringaldehyde, syringic acid, vanillin and ferulic acid which are inducers of *Agrobacterium* virulence genes. Some parasites seem to have developed a detoxification mechanism which involves the catabolic enzymes to defend against phenolic prohibitins. Hence detoxification of phytoalexins has been implicated in host specificity (MAHADEVAN, 1979).

ROLE OF GLYCOSIDASE & CELL DEGRADING ENZYMES IN VIRULENCE INDUCTION

Wounding and plant cell wall disruption by glycosidases and enzymes like cellulase release free monosaccharides and oligosaccharides which elicit phytoalexin and other secondary metabolites, and *vir* induction ensues (ANKENBAUER & NESTER, 1990). Phenols do not exist in plant as such but do as glycosides. Glycosides are mobile but non-toxic, whereas aglycones are immobile and toxic. Aglycones have been well documented as the inducers of virulence in *Agrobacterium* (VICKERY & VICKERY, 1981).

Wounding is a prerequisite for transformation by *Agrobacterium*. Immediately upon wounding, cellular decompartmentalization of glycosidases takes place and the glycosides are cleaved (MAHADEVAN, 1991). *Vir* gene activation by aglycones is the crucial one, but is a secondary event consequent to glycosidase activity.

In *Pseudotsuga menziesii*, coniferin, a phenylpropanoid glucoside is an efficient inducer of virulence in gymnosperm-specific *A. tumefaciens* strains, which possess B - glucosidase activity. B - glucosidase converted coniferin into the proximal inducer coniferyl alcohol (MORRIS & MORRIS, 1990).

Aldoses with C-3 stereochemical structures like L-arabinose, D-xylose, D-glucose, D-galactose are effective *vir* gene inducers. The active sugars even at concentrations lower than 1mM may effect *vir* inducing activity, by phenolic compounds but in combination with other extracellular prevailing conditions like pH and temperature. Another interesting finding is the *vir* gene induction by synergistic action of weak phenol inducers with monosaccharides (SHIMODA *et al.*, 1990). D-Galacturonic acid, a monosaccharide is a strong *vir* inducer even at low concentration. It is a product of polygalacturonase of *A. tumefaciens* bv. 3 strain specific for grapevine (RODRIGUEZ-PALENZUELA *et al.*, 1991).

Cellobiose, a breakdown product of cellulose, is an active inducer (CANGELOSI *et al.*, 1990). Hence, investigations on enzymes degrading cell wall components like cellulose, galactans, xyloglucans with respect to the inducing activity of breakdown products may give insight into the *Agrobacterium* interaction with plants.

Therefore to understand *vir* induction, its role in pathogenesis of *Agrobacterium*, host specificity among dicots and the recalcitrant nature of monocots, the following questions require investigations :

1 What is the role of glycosidases, cell wall degrading enzymes of the bacterium and plants ?
2 What are the concentrations of aglycones and sugars at the wound sites ?
3 How are *vir* genes induced under *in vivo* condition by aglycones ?
4 Are the glycosides mobilized before the glycosidases act on them to generate *vir* inducers in different tissues ?
5 Is localized *vir* induction sufficient to trigger tumor formation ?
6 What is the minimum level of induction of *vir* genes needed for efficient transformation ?

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Role of toxins and polysaccharides in bacterial pathogenesis

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Recently, several laboratories concentrated their efforts to answer the question why phytopathogenic bacteria can **not** cause disease in certain combinations, such as those leading to induction of the hypersensitive response by the *hrp* genes in non-host plants or by the so-called avirulence genes in incompatible cultivars. However, the mere absence of a resistant reaction does not necessarily explain how and why the disease develops during the susceptible reaction. In order to induce disease, bacteria need specific capabilities, the factors for pathogenicity and/or virulence.

A large group of virulence factors is comprised of **toxins.** Toxins can be defined as non-enzymatic products of a pathogen, which are harmful to plants in low concentration. Bacterial polysaccharides, on the other hand, occur in rather high concentration in the infected plant and thus may not be regarded as toxins. However, polysaccharides also appear to be important factors of virulence and are therefore included in this review.

Often, it has been rather difficult to assess whether certain products of bacteria play a role during pathogenesis. In future, these questions may be resolved by genetic analysis. However, I want to point out that still possibilities for patho-physiological experiments exist which may also support or disprove whether a toxin plays a role in pathogenesis. Such criteria for assessment of the pathogenic role of toxins may be:

- Typical disease symptoms are induced by the purified toxin.

- Toxin can be demonstrated in the diseased plant.
- Toxin production *in vitro* correlates with virulence of the bacterial strain.
- Disease susceptibility of different cultivars correlates with toxin sensitivity.
- Toxin production on resistant cultivars is lower than on susceptible ones.
- Toxin can break disease resistance.

The first 3 criteria should be positive if a toxin plays a role in pathogenesis, the other 3 criteria are not obligatory.

In the case of the leaf spot causing bacteria, many pseudomonads produce low-molecular weight toxins, often related to peptides and related molecules, e.g. tabtoxin, coronatine, phaseolotoxin, tagetitoxin (Mitchell 1984, Rudolph 1990). Another example of this group is rhizobitoxine which is not only produced by rhizobia but also by *P. andropogonis*. The structures of the syringomycins have been recently elucidated (Gross 1991). A similar toxin is produced by the mushroom pathogen *P. tolaasii* (Brodey et al. 1991).

Only recently **molecular genetic analyses** involving site-specific mutants have been carried out to investigate the contribution of particular toxins to virulence. In the case of syringomycin, genetic studies revealed that Tox$^-$-mutants formed smaller necrotic lesions in immature cherry fruits (Xu and Gross 1988). The *syrB* gene required for syringomycin production by *Pseudomonas syringae* pv. *syringae* was transcriptionally activated within 24 h of inoculation (Mo and Gross 1991). Nevertheless, syringomycin production did not stimulate bacterial growth *in planta* in these systems (Gross 1991), although the toxin accentuated the disease by killing a larger number of host cells. Mutants of *P. syringae* pv. *tomato* defective in coronatine production caused necrotic lesions in tomato leaves that were only about one third the size of those produced by the wild-type strain (Bender et al. 1987). Also, the multiplication of Tox$^-$-mutants was

significantly reduced. For tabtoxin genetic studies of Kinscherf et al. (1991) revealed that all Tox⁻-mutants were indistinguishable from parental strains in ability to grow *in planta*. This result is also underlined by the occurrence of natural Tox⁻-forms of *P. syringae*, designated as pv. "*angulata*" and pv. "*striafaciens*" (Willis et al. 1991).

Different **mode of actions** appear to play a role during pathogenesis of bacterial toxins. For several years we studied the chlorosis-inducing toxin of *P.s.* pv. *phaseolicola*, called phaseolotoxin (Moore et al. 1984). Interestingly, only very rarely have naturally occurring toxin-negative strains of this organism been described. All known toxin-negative strains are characterized by a comparable low aggressiveness in the field. The toxin inhibits ornithine transcarbamylase and other metabolic processes in the plant which result in suppression of resistance mechanisms (Rudolph 1976), even against other bacterial pathogens of bean (Rudolph 1972).

The toxin coronatine is produced by several pathovars of *P. syringae*, especially *tomato* and *glycinea*. The exact mode of action is unknown. The initial toxin-caused chlorosis may turn to necrosis in later stages as shown on soybean leaf after natural infection (Abo-Moch et al. 1991). Thus, the toxin may lead to release of nutrients by causing membrane-damage in the plant tissue. However, in the case of coronatine, toxin-negative strains are quite commonly isolated, indicating that this toxin may not be very important in pathogenesis (Abo-Moch et al. 1991).

Similarly, syringomycins may enhance disease by causing membrane damage (Iacobellis et al. 1993). Also, the antibiotic effect of the syringomycins is very striking. Since this group of pseudomonads has a well-documented epiphytic growth phase, the antibiotic effects of the syringomycins towards other leaf-colonizing microorganisms can be very decisive in the epidemic of these bacteria. Induction of membrane leakage has also been reported for tolaasin. A tolaasin defective mutant of *Pseudomonas tolaasii*

was not able to disrupt the plasma membrane and vacuole membranes of the host mushroom *Agaricus bisporus* in contrast to the wild strain (Rainey et al. 1991). The culture filtrate of the mutant also was unable to pit mushroom tissue and to lyse erythrocytes.

Toxin effects can also be induced by secondary metabolites of the bacteria without playing a decisive role during pathogenesis. This may be the case for several carboxylic acids produced by *Xanthomonas campestris* pathovars. It was recently shown by Ewbank and Maraite that two bacterial enzymes synthesize carboxylic acids from amino acids by a transamination and decarboxylation reaction. It was suggested that the blight symptom induced by these bacteria is caused by a cumulative toxic effect of several carboxylic acids (Ewbank and Maraite 1990a and b, Ewbank 1992).

The phytotoxin dihydrophenylalanine is only produced by a few strains of *E. amylovora* and absent in many virulent strains (Schwartz et al. 1991). Therefore, Geider et al. (1990) suggested that dihydrophenylalanine, which is not a general virulence factor of this organism, may favour the producing strains by inhibiting competiting microorganisms and damaging plant cells.

Related to the toxins is a second group of bacterial virulence factors, the **extracellular polysaccharides (EPS)**. EPS are assumed to be responsible for the water-soaking symptom in bacterially infected plant tissue (Rudolph et al. 1989). Water-soaking is necessary for maximum bacterial multiplication in many cases (e.g. Rudolph 1984, El-Banoby and Rudolph 1989). Light and electron microscopy revealed masses of bacteria which filled the intercellular spaces of the leaf mesophyll and were embedded in a network of acidic EPS (Rudolph et al. 1987). Xanthomonads produce the acidic exopolysaccharide xanthan (Sutherland 1977, Miles et al. 1991), and many *P. syringae* pathovars synthesize alginate, a polymer of acetylated mannuronic acid which may contain some guluronic acid (Gross and Rudolph 1984, 1987a, Fett et al. 1986). The major difference from algal material is the

presence in bacterial alginates of O-acetyl substituents. These are readily removed by mild alkaline treatment. The O-acetyl content is apparently proportional to the mannuronic acid content. From this it was inferred that the mannuronosyl residues were acetylated (Sutherland 1977). The conditions for alginate production *in vitro* and the characterization of the product are described in 3 contributions of these proceedings (Schade 1993, El-Shouny et al. 1993, Sonnenberg et al. 1993).

Two different polysaccharides are produced by *Erwinia amylovora*, the acidic heteropolysaccharide amylovorin, also called amylovoran (Smith et al. 1990, Geider et al. 1993), and the neutral levan (Gross et al. 1992). Genetic studies of Geider et al. (1991) revealed that amylovorin-deficient strains were avirulent and induced phytoalexin and callose production in plant cells. Mutants with a levan-deficiency showed some retardation of symptom development on pear seedlings. It was suggested that bacterial cells not surrounded by EPS come in contact with plant cell walls and, therefore, induce the HR.

The neutral levan is also synthesized by many phytopathogenic pseudomonads (Hettwer et al. 1993). Bacterial levan as well as alginate could be demonstrated *in planta* (Gross and Rudolph 1987b). We concluded that more than 5% of the total dry weight of infected bean leaf tissue was bacterial EPS. Taking into account the different percentages of dry mass compared to the fresh weights of bacterial slime (1-2%) and plant tissues (c. 15%), it becomes evident that in severely infected tissues probably 1/4 to 1/3 of the fresh weight are bacterial slimes. This means that the normally air-filled intercellular space actually is completely filled with EPS. This consideration could explain two observations made with infected plants: (1) the formation of exudates, which seem to be extruded out of the tissue likewise "under pressure", and, (2) the observation that infected, necrotized tissue for several days retains a remarkably higher weight than non-infected one.

In addition to EPS the **lipopolysaccharides (LPS)** may play a role during pathogenesis. Although LPS are constituents of the outer membrane of Gram-negative bacteria, they are also released into the surrounding medium (Ramm 1991). That the O-specific side chain of the LPS can be very specific (Gross et al. 1988, Yakovleva et al. 1993) is of interest. Bacterial mutants of *P.s.* pv. *phaseolicola* which were deficient in the O-specific side chain proved to be non-pathogenic. EM-photos of these bacteria *in planta* revealed that bacteria were very densely packed (Rudolph et al. 1989) without any space of EPS between them. Thus, these bacteria were obviously not able to grow into the intercellular space.

Recently, Wydra et al. (1993) characterized a high molecular weight polysaccharide from resistant bush bean leaves, which agglutinated intact bacterial cells and precipitated LPS fractions. The bacterial EPS counteracted this resistance-like reaction.

In conclusion, five possible functions of toxins and polysaccharides during pathogenesis are suggested:

a) release of nutrients (e.g. syringomycins, tolaasin, coronatine, tabtoxin, lipopolysaccharides);

b) suppression of plant resistance (e.g. phaseolotoxin, tagetitoxin, tabtoxin, coronatine, rhizobitoxine);

c) creation of a favourable micro-environment (e.g. attraction of water, closure of stomata, alkalization, gall formation, chelation of metals);

d) facilitation of epidemic spread (e.g. protection from desiccation by EPS); and

e) antibiotic effects towards other microorganisms (e.g. syringomycin).

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Exopolysaccharides in pathogenicity of *Erwinia amylovora*

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ABSTRACT

The fireblight pathogen *Erwinia amylovora* produces two major exopolysaccharides, the heteropolymer amylovoran and the fructose homopolymer levan. The levansucrase gene was mutated, cloned and sequenced. There is some homology to the levansucrase gene of *Bacillus subtilis*. In *E. amylovora* it is constitutively expressed and Tn5-mutants in that gene are retarded for spread of *E. amylovora* on sucrose-containing plant tissue.

A chromosomal region with genes for amylovoran synthesis was characterized by transposon mutagenesis. Genes for amylovoran-synthesis (*ams*) were localized on a cloned fragment and partially sequenced. Transcription in the *ams*-region is from left to right and started at individual genes. All mutants deficient in amylovoran synthesis did not produce fireblight symptoms on immature pears unless they were complemented with intact genes. Pathogenicity EPS-mutants can also be restored by cocultivation with other pathogenicity mutants of *E. amylovora* deficient in genes apart from EPS-synthesis.

Mutants in the *gal*E-gene lack synthesis of EPS in the absence of galactose indicating a role of UDP-galactose as a precursor in amylovoran-synthesis.

Keywords: fireblight, transposon mutants, levan, levansucrase, amylovoran

INTRODUCTION

Fireblight is a disease of pome fruit trees and other Rosaceae. Disease symptoms are watersoaking and necrosis and most strikingly ooze production in infected tissue of host plants. EPS produced can be either levan, a homopolymer of fructose residues (GROSS et al., 1992) or the acidic amylovoran (HSU & GOODMAN, 1978; BENNETT & BILLING, 1980). Pathogenicity mutants with Tn5-insertions are mainly affected in genes connected to the hypersensitive reaction (HR) of non-host plants (STEINBERGER & BEER, 1988; BARNY et al., 1990) or in genes involved in biosynthesis of exopolysaccharides (STEINBERGER & BEER, 1988; BELLEMANN & GEIDER, 1992). Regulatory genes of EPS synthesis like the *rcsA* gene also influence virulence of *Erwinia amylovora* (BERNHARD et al., 1990).

RESULTS and DISCUSSION

Characterization of the levansucrase gene.

Levan is synthesized by the enzyme levansucrase (β-2,6-D-fructan:D-glucose 6-fructosytransferase; E.C.2.4.1.10.), which cleaves sucrose with concominant polymerization of the fructose residues (GROSS et al., 1992). Levansucrase from *E. amylovora* is expressed constitutively independent from the carbon source and secreted into the medium. The purified enzyme has a molecular weight of 46 kD on SDS gels and produces two bands with isoelectric points at pH 4.0 and 3.6. The structural gene was mutagenized with transposon Tn5. Besides a lack of enzyme secretion the mutants produced retarded symptoms on pear seedlings, but they were unchanged in ooze production on slices of immature pears. It appears that levan synthesis is important to spreading of the pathogen on plant tissue containing sucrose in order to create quickly a barrier of protective exopolysaccharides. By complementation of the mutant with a genomic library of *E. amylovora* DNA a chromosomal fragment encoding levansucrase activity was obtained, which was subcloned to a *Pst*I-*Eco*RV fragment of 1.4 kb. The gene was constitutively expressed in *E. coli*, and the enzyme was predominantly localized in the periplasm. In *E. amylovora* levansucrase is secreted into the medium, and very little activity was found to be associated with the cells. The cloned DNA-fragment was sequenced in both strands. The levansucrase gene was preceeded by a consensus sequence for a

bacterial promoter and a ribosome binding site. The nucleotide sequence derived from the structural gene corresponded to a protein of 46 kD in agreement with the purified protein on SDS gels. Homology on the amino acid level exists to levansucrase of *Bacillus subtilis* (30 %) and to fructosyltransferase of *Streptococcus mutans* (20 %). The deduced amino acid sequence does not contain a hydrophobic signal at the N-terminus. Attempts to determine parts of the amino acid sequence failed presumably due to a protected N-terminus (unpublished). Considering the close correspondence of the molecular weight calculated from the sequence of the gene and its molecular weight on SDS gels, it appears that the protein is synthesized without or with a small signal peptide and exported through the inner membrane without detectable cleavage of a signal peptide.

Genetics of EPS-synthesis.

Transposon mutagenesis with various vectors (BELLEMANN & GEIDER, 1992; METZGER et al., 1992) produced strains with defects including a lack in synthesis of the acidic EPS amylovoran. The mutations were localized on *Eco*RI-fragments of various sizes. In another approach EPS-mutants of *Erwinia stewartii* were complemented with a genomic library of *E. amylovora* and a 15.5 kb insert in vector pVK100 was obtained which complemented also many of the Tn5 mutants of *E. amylovora* (BERNHARD et al., 1993a and b). A map obtained from genetic complementation was established (BERNHARD et al., 1993a,b). Tn5 mutagenesis of this region narrowed genes involved in EPS-synthesis to about 9 kb. This region (*ams)* was subcloned and the fragments used for sequence analysis. Fusions with reporter genes were expressed from left to right transcription of the *ams*-region (Fig. 1). No significant influence of the *rcsA* gene (BERNHARD et al., 1990) on these fusions has been found. A mutant in the *galE*-gene lacked galactose-epimerase (METZGER et al., 1992), which could be involved in synthesis of the repreating unit, the precursor of the exopolysaccharide formed. The *galE*-gene was located apart from the *galK,T*-genes. The deficiency of the mutant in EPS-synthesis was suppressed by addition of galactose to the medium and to pear slices. A mutant in the *dsp*-region was able to complement *ams*-mutants of *E. amylovora* on pear slices (BELLEMANN & GEIDER, 1992). The capsule may be loosely associated with the bacterial cells, and the EPS can diffuse to non-capsulated cells and also protect these against plant defense mechanisms.

Sequence analysis of ams-genes.

The dideoxy method was used for sequencing the *ams*-region of *E. amylovora*. At least six open reading frames (ORFs) were found, which closely corresponded to the genetically assigned *ams*-genes. They were preceeded by sequences typical for ribosome binding sites. A promoter sequence similar to the consensus of *E. coli* was visible in front of gene *ams*A. All ORFs are closely packed with very short spacing sequences separating them. Non-polar transposon insertions and complementation studies suggest individually translated genes in the *ams*-region. A comparison of the genetic map and these data is presented in Fig. 1. The *gal*E-gene of *E. amylovora* was not present in plasmid pEA109. It seems to be located adjacent to the right side of the *ams*-genes. Plasmid pES2144 carries genes for EPS-synthesis of *E. stewartii* together with the *gal*E-gene (see BERNHARD et al., 1993b) suggesting a relation between galactose epimerase and EPS-synthesis of that pathogen, too. Sequence analysis of the a DNA fragment with the *gal*E-gene of *E. amylovora* showed a high degree of homology to the *gal*E-gene of *E. coli*.

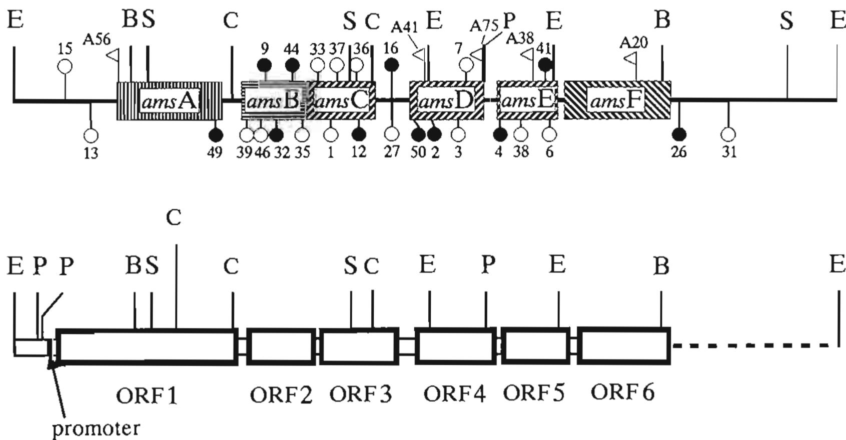

Fig. 1: Comparison of genetic mapping and sequence data. Circles: Tn5 insertions in pEA109; filled circles and triangles: Chromosomal Tn5 insertions in *E. amylovora*. Cleavage sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I. The inner restriction sites in the lower part were deduced from sequence data, whereas the sites in the upper part of the map were derived from physical mapping.

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A pathogenicity gene of *Pseudomonas syringae* pv. *syringae* complements a defect in periplasmic glucan biosynthesis in *Escherichia coli* K-12

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SUMMARY: The *mdoH* gene of *Escherichia coli* encodes a membrane-bound glucosyl transferase implicated in periplasmic glucan biosynthesis. ORF2 of the *hrpM* pathogenicity locus of *Pseudomonas syringae* pv. *syringae* is required for growth of bacteria *in planta*. A multicopy plasmid bearing the *hrpM* locus restored periplasmic glucan synthesis when introduced into an *mdoH* strain of *E. coli* . Thus, the pathogenicity function of the *hrpM* locus appears related to periplasmic glucan synthesis.

KEYWORDS: *E. coli, P. syringae*, periplasmic glucans, pathogenicity.

Periplasmic glucans are general components of Gram-negative bacteria and share the following features: i) glucose as the sole sugar; ii) glucose units linked (at least partially) by β,1-2 glycosidic bonds; iii) synthesis under osmotic control and inversely proportional to osmolarity; iv) certain sugar units substituted by *sn*-1-phosphoglycerol originating from membrane phospholipids.

Among members of the family *Rhizobiaceae*, periplasmic glucans are cyclic. They are thought to play an important role in the interaction of bacteria with specific plant hosts (DOUGLAS *et al.*, 1985; GEREMIA *et al.*, 1987). The molecular basis of this function remains poorly understood. Mutations in the glucan biosynthesis genes (*ndvA* and *ndvB* in *Rhizobium*, *chvA* and *chvB* in *Agrobacterium*) do not confer a particular phenotype to the bacteria except in their interaction with plant hosts.

Membrane-derived oligosaccharides (MDO) which are present in the periplasm of *Escherichia coli* represent 5-7% of the dry weight of bacteria grown in a medium of low osmolarity (KENNEDY, 1987). Despite this fact, growth in a medium of low osmolarity of mutant strains unable to synthesize MDO (Mdo$^-$) is not significatively affected (BOHIN & KENNEDY, 1984). Thus MDO do not appear to be components essential for bacterial growth under laboratory conditions.

Two genes implicated in the biosynthesis of MDO were recently described as forming an operon (*mdoGH*) whose transcription is under osmotic control. Complete nucleotide sequencing of this operon and gene-fusion experiments revealed that MdoG is located in the periplasmic space while MdoH crosses the cytoplasmic membrane (LOUBENS *et al.*, to be published elsewhere). MdoH is known to be necessary for a specific glucosyl-transferase activity (LACROIX *et al.*, 1991), but the function of MdoG is unknown.

Comparison of the nucleotide sequence of the *mdoGH* operon (4759 bp) with various data bank entries (DESSEN *et al.*, 1990) revealed a striking homology between *mdoGH* and the *Pseudomonas syringae* pv. *syringae* locus *hrpM* (for Hypersensitive Reaction and Pathogenicity). *Pseudomonas syringae pv. syringae* is the causal agent of brown spot disease of *Phaseolus vulgaris*. Genes at this locus are required for both the expression of disease symptoms on host plants and the development of the hypersensitive reaction on nonhost plants (Mills & Mukhopadhyay, 1990). The *hrpM* gene product is not required for growth in minimal culture medium but is required for growth of bacteria *in planta*.

As shown in Figure 1, two regions of strong homology can be observed. Like *mdoGH*, *hrpM* consists of two ORFs organized as an operon (Mukhopadhyay *et al.*, 1988). Each ORF of *P. syringae* contains a stretch of about 69% homology with the corresponding gene of *E. coli*. The central regions differ in size and do not share homology. This high homology suggests that these genes could have similar functions in both organisms.

To test this hypothesis, we attempted to complement the defect in MDO synthesis of a *mdoH* strain of *E. coli* by plasmids harboring *hrp* genes.

The common vector was pYZ4, a kanamycin resistant plasmid which allows the expression of heterologous genes under the control of the *lac*UV5 promoter (BROOME-SMITH *et al.*, 1990). The 3.1 kb *Mlu*I-*Ssp*I fragment of

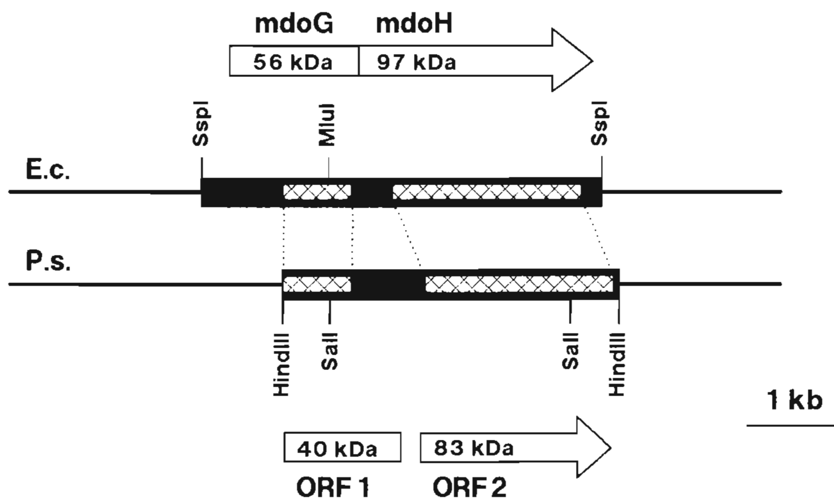

Fig.1. Genetic structure of the homolous regions of the chromosomes of *E. coli* and *P. syringae* pv. *syringae*. Black boxes represent the regions of each chromosome whose nucleotide sequences are known. Hatched boxes represent the regions where the nucleotide sequences are 69 % identical between both organisms. The direction of transcription is shown by arrows; the limits of ORFs and the sizes of putative polypeptides encoded by these ORFs are indicated.

mdoGH and the 3.9 kb *Hin*dIII fragment of *hrpM* were cloned into the polylinker site of pYZ4, downstream of the *lac*UV5 promoter. The resulting plasmids, pNF309 and pNF331 respectively, and the unmodified vector were introduced by transformation into strain NFB213 (*pgi*::Mu, Δ(*zwf-edd*)1, *mdoH200*::Tn*10*; LACROIX *et al.*, 1991). Specific labelling of MDO eventually produced in these various transformants was obtained by growing the cells in the presence of radioactive glucose. Mid-log cultures in low-osmolarity medium were submitted to the charcoal adsorption procedure (KENNEDY, 1982) and aliquots of the extracts were analysed further by chromatography on Sephadex G-25 as previously described (LACROIX *et al.*, 1991).

Bacteria harbouring plasmid pNF331 (*hrpM*$^{+}$) were able to produce glucans, and these oligosaccharides seemed similar in size to those produced in bacteria harbouring plasmid pNF309 (*mdoH*$^{+}$), as judged by this preliminary characterization (Figure 2). Moreover, the reduction in MDO

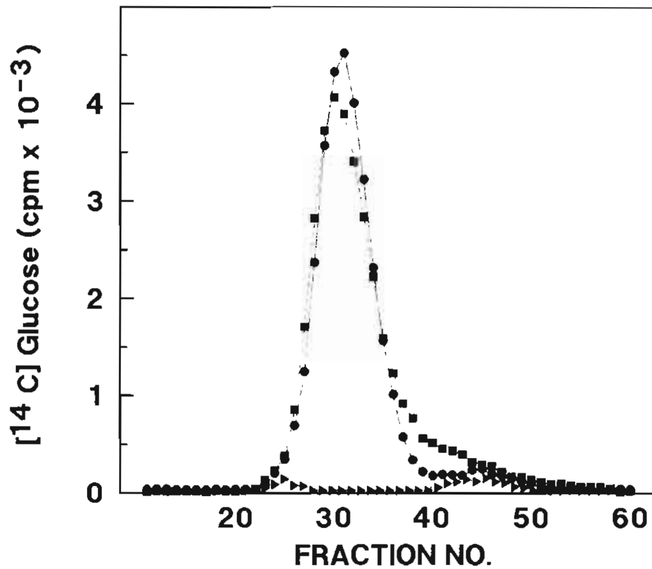

Fig.2. Chromatography on Sephadex G-25 of extracts from cultures labeled with [^{14}C]-glucose. Extracts were of cells of NFB213 harbouring plasmid pYZ4 (triangles), pNF309 (*mdoH*$^+$, circles) or pNF331 (*hrpM*$^+$, squares) grown in 5 mL of low-osmolarity medium supplemented with kanamycin (50 μg/mL) and 0.24 mM D-[U-^{14}C]-glucose (125 MBq mmol^{-1}).

biosynthesis produced by the addition of 0.3 M NaCl to the low-osmolarity medium was identical in both strains (data not shown).

Thus, it appears that ORF2 of the *hrpM* locus of *P. syringae* pv. *syringae* very likely encodes a membrane-bound glucosyl transferase and that a defect in this activity is responsible for the particular phenotype of *hrpM* mutants. A complete demonstration of this fact requires that the non-pathogenic phenotype of a *hrpM* strain of *P. syringae* be reversed upon introduction of the *mdoGH* operon from *E. coli*, when introduced into . Moreover, further confirmation needs the demonstration that *hrpM* mutants are defective in some kind of periplasmic glucans. Both kinds of experiments have been undertaken in our laboratories.

The functional homology between genes implicated in periplasmic glucan biosynthesis in *E. coli* (which colonizes animals) and pathogenicity in *P. syringae* (which colonizes plants) stongly supports the assumption that

periplasmic glucans are general components of the envelope of Gram-negative bacteria essential for the colonization of their hosts.

RESUME : Le gène *mdoH* d'*Escherichia coli* code une glucosyl-transférase membranaire impliquée dans la biosynthèse des glucanes périplasmiques. L'ORF2 du locus de pouvoir pathogène *hrpM* de *Pseudomonas syringae* pv. *syringae* est requise pour la croissance des bactéries dans les plantes. Un plasmide à copies multiples, portant le locus *hrpM*, permet de rétablir la synthèse des glucanes périplasmiques lorsqu'il est introduit dans une souche *mdoH* d'*E. coli*. Ainsi, la fonction du locus *hrpM* dans la pathogenèse apparait liée à la synthèse de glucanes périplasmiques.

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Characterization of a gene involved in cytokinin biosynthesis in *Pseudomonas amygdali*

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ABSTRACT

A plasmid-borne cytokinin gene of *Pseudomonas amygdali* strain NCPPB2610 cross-hybridized with the isopentenyl transferase (*ipt*) gene from *P. syringae* subsp. *savastanoi.* Sequencing analysis showed that the two genes share a 95% homology at nucleotide level and a 97% homology at aminoacid level.

Pathogenicity tests performed on almond with *P. amygdali* strains producers of different levels of cytokinins (NCPPB2610 and its mutant NCPPB2610-1) showed that cytokinins could play an important role in the hyperplastic bacterial canker disease of almond.

INTRODUCTION

Recent studies have shown that *P. amygdali*, a pathogen of almond, secretes high level of auxins and cytokinins in culture (IACOBELLIS *et al.*, 1988) and this production seems to be positively correlated to the virulence of the producing strains (IACOBELLIS *et al.*, 1990b).

All the *P. amygdali* strains so far examined have a complement of 3 to 4 plasmids ranging from 79 to 31 kb (IACOBELLIS *et al.*, 1991). Strain NCPPB2610, which accumulates high level of auxins and cytokinins in culture, contains four plasmids with molecular sizes of 79, 70, 69 and 36 kb. Its spontaneous mutant NCPPB2610-1, a producer of very low level of cytokinin in culture, lacks the 69 kb plasmid which was shown to harbour sequences homologous to the isopentenyl transferase (*ipt*) gene from *P. syringae* subsp. *savastanoi* (IACOBELLIS *et al.*, 1990a)

Here we report on the characterization of a gene involved in cytokinin

biosynthesis in *P. amygdali* and on its role in the disease.

MATERIAL AND METHODS

Genomic-DNA was isolated by the procedure of Glass and Kosuge (1988); plasmid-DNA was obtained as previously reported (IACOBELLIS *et al.*, 1991).

DNA was digested with restriction enzymes as indicated by the manufacturer (Boehringer Mannheim GmbH, Biochemica, Italy) and analysed by 0.5 or 0.7% agarose gel electrophoresis in Tris-Borate buffer.

DNA was transferred from agarose gel to nitrocellulose filters following Southern's procedure (SOUTHERN, 1975) and hybridized with the 0.6 kb RsaI fragment of *ipt* gene from *P. s.* subsp. *savastanoi* (POWELL and MORRIS, 1986) labeled by using the "Random primer method"(FEINBERG and VOGELSTEIN, 1983).

Plasmid-DNA from *P. amygdali* strain NCPPB2610 was digested with appropriate restriction enzymes and the DNA-fragments were separated by agarose gel electrophoresis. Fragments showing cross-hybridization with the *ipt* gene were identified, isolated from agarose gels and ligated to linearized vector pUC19 (NORRANDER *et al.*, 1983). The above ligation mixtures were used to transform *Escherichia coli* TGI.

Recombinant plasmids which showed cross-hybridization with the probe were isolated by standard procedures (MANIATIS *et al.*, 1982).

The dideoxy chain-termination method (SANGER *et al.*, 1977) was used with Sequenase Version 2.0 (U.S. Biochemical) enzyme. Computer analyses were performed on the VAX computer at the EMBnet node at Tecnopolis (Valenzano, Bari, Italy).

Genomic-DNA was amplified using the Perkin Elmer Cetus GeneAmp PCR System 9600; 35 cycles of amplification were performed between 94°C and 65°C.

The virulence and the cytokinin production of *P. amygdali* were evaluated as previously reported (IACOBELLIS *et al.*, 1990b).

RESULTS AND DISCUSSION

The digestion of plasmid DNA from *P. amygdali* strain NCPPB2610 with several restriction enzymes showed that DNA sequences homologous to the *ipt* gene from *P. s.* subsp. *savastanoi* were located on a BglII DNA fragment of

about 3.0 kb.

The transformation of *E. coli* TG1 with the ligation mixture (pUC19 plus 3.0 kb BglII electroeluted fragment) gave rise to two *ipt* cross hybridizing clones. However, the restriction analysis of the clones (pITM27, pITM51) showed the insertion of an extra fragment of DNA of about 700 bp identical in both clones. The sequencing analysis of pITM27 confirmed that the inserted fragment was 633 bp in length. When we aligned the sequences from *P. amygdali* and those of the *ipt* gene from *P. s.* subsp. *savastanoi* (POWELL and MORRIS, 1986) we found that the inserted fragment was an abbreviated DNA coding region. In particular, it was lacking 72 nucleotides at the 5' DNA coding region and terminated with the stop codon. However, they showed a 95% homology at nucleotide level and a 97% homology at aminoacid level.

The above findings and the fact that no clone containing the entire gene was obtained may be due to the instability of this gene in plasmid vectors based on the ColE1 replicon as suggested by Powell and Morris (1986) for the *ipt* gene from *P. s.* subsp. *savastanoi* . This hypothesis is supported by the finding that other clones containing abbreviated 5' DNA coding region fragments were, on the contrary, stably maintained in pTZ19R, another plasmid vector based on the same replicon.

A PCR-mediated amplification of DNA from *P. amygdali* strain NCPPB2610, using two oligonucleotides from regions spanning the start and stop codons of the cytokinin biosynthetic genes from *P. s.* subsp. *savastanoi* and *P. amygdali*, respectively, confirmed that the two genes were almost identical.

When *P. amygdali* strain NCPPB2610 and its mutant NCPPB2610-1, which lacks the cytokinin biosynthetic gene, were assayed for pathogenicity on almond, they showed different levels of virulence. The mutant, which produces the same level of auxins as the parental strain and a low amount of cytokinins as determined by HPLC, induced hyperplastic cankers of reduced size in comparison to the parental strain. These findings clearly demonstrated that cytokinins, as ascertained for *P. s.* subsp. *savastanoi* (SURICO *et al.*, 1985; SISTO *et al.*, 1991), are not essential for the pathogenicity of *P. amygdali*, but they do play a significant role in the amplitude of disease symptoms.

The almost identical sequences of the cytokinin biosynthetic genes in *P. amygdali* and *P. s.* subsp. *savastanoi*, both inducers of hyperplastic symptoms on their respective host plants, are noteworthy and suggest a common ancestral origin.

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Race-specific conservation of genes required for coronatine production of *Pseudomonas syringae* pv. *glycinea* and correlation to copper resistance

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ABSTRACT

Production of the phytotoxin coronatine and copper resistance correlated in 19 field-isolates of *Pseudomonas syringae* pv. *glycinea* and 28 strains from a culture collection. All of the coronatine-producing isolates were representative for races 4 and 6 of this pathovar. Diversity in *P.s.glycinea* was investigated by plasmid profiling, by DNA hybridization with plasmid pSAY1 which carries genes involved in coronatine synthesis (BENDER *et al.*, Appl. Environm. Microbiol. 57 (1991) 993-999), and by PCR analysis with a random primer. Strains which did not produce coronatine were sensitive to copper ions and showed no homology to plasmid pSAY1. They could be distinguished from coronatine-producing strains by the absence of a PCR-band. A common profile of other PCR products in both types of *P.s. glycinea* enabled us to classify coronatine-producing and non-producing strains as pv. *glycinea* and to differentiate them from strains from other pathovars tested. Plasmid patterns of strains isolated in a soybean field revealed conservation of the plasmid content in isolates from 1983, 1985, and 1990 and diversity of plasmid profiles between coronatine producers and non-producers. The plasmid profiles vary in 28 strains from the culture collection. No correlation of plasmid pattern and race specificity was observed. Coronatine-producing strains shared homology with plasmid pSAY1 and also showed RFLPs of the flanking segments of a plasmid DNA region involved in coronatine synthesis.

Keywords: bacterial blight, phytotoxin, PCR, races, plasmid profile

INTRODUCTION

Pseudomonas syringae pv. *glycinea* (*P.s. glycinea*), the causative agent of bacterial blight of soybean, can produce the non-host specific phytotoxic polyketide coronatine which induces chlorosis, hypertrophy and stunting of plant tissues. Several reports have shown that synthesis of the phytotoxin enhances virulence of bacteria and therefore contributes possibly to the biological fitness of the pathogen *in planta* (BENDER *et al.*, 1987; GNANAMANICKAM *et al.*, 1982; SATO *et al.*, 1983). Only little is known about natural field-populations of *P. syringae* with respect to coronatine production. Genes required for coronatine production had been linked to plasmid DNA in at least three pathovars (SATO *et al.*, 1983; BENDER *et al.*, 1989; YOUNG *et al.*, 1992) and conservation of a cloned 30 kb DNA region (pSAY1), which was derived from plasmid pPT23A (101 kb) of *P.s. tomato* strain PT23.2, was found in large plasmids of coronatine producing strains of other pathovars of *P. syringae* (BENDER *et al.*, 1991). Recently, a region of the 90 kb plasmid p4180A of *P.s. glycinea* strain PG4180 was shown to be required for coronatine production. Furthermore, mutants of PG4180 lacked distinct biosynthetic steps and could be complemented by feeding experiments (YOUNG *et al.*, 1992). In *P.s. glycinea*, little information is available about correlations of phytotoxin production, copper resistance and affiliation to races. In this study, we determined the occurrence of phytotoxin production for a number of field isolates of *P.s. glycinea* using a bioassay, and we analyzed plasmid profiling of the bacteria, hybridization of plasmid DNA to a DNA-probe carrying genes for coronatine synthesis from *P.s. tomato* (BENDER *et al.*, 1991), and affiliation of the strains to races. A PCR amplification assay with arbitrarily synthesized oligonucleotides (WILLIAMS *et al.,* 1990) enabled us to differentiate coronatine producing and non-producing strains of *P.s. glycinea*.

RESULTS

Coronatine production of P.s. glycinea strains

From soybean plants cv. Maple Arrow, 76 strains of *P.s. glycinea* were isolated in the field during the vegetation period in 1990. The strains were screened for their ability to cause hypertrophy on potato tuber discs (VÖLKSCH *et al.*, 1989). Out of the 76 isolates 23 failed to produce coronatine. Coronatine-producing (cor^+) and non-producing (cor^-) strains were isolated throughout the season (Fig. 1). Seven cor^+ and nine cor^- strains were randomly selected for further genetic studies. *P.s. glycinea* strains from various geographic origins,

which were derived from the Göttinger Sammlung Phytopathogener Bakterien (GSPB), represented all races. Half of the 28 GSPB-strains tested did not synthesize the phytotoxin. Cor+ GSPB strains were exclusively of races 4 or 6. Three strains of race 4 were cor−.

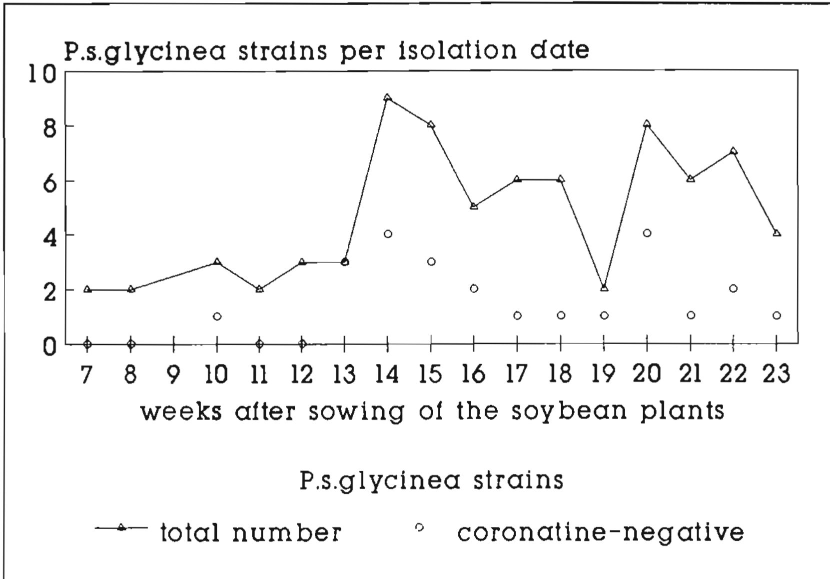

Fig. 1. Proportion of coronatine-negative P.s. glycinea strains in 1990-isolates of a soybean field.

Plasmid profiles of various cor+ and cor− P.s. glycinea strains

The plasmid DNA content from 16 field strains of 1990 and three other cor+ *P.s. glycinea* strains isolated in 1983 and 1985 from the same soybean field was investigated. Plasmids could be grouped into four size classes ranging from 70 - 100 kb (class A), 30 - 60 kb (class B), 11 - 15 kb (class C), and 8 kb (class D). Plasmids of a subclass of class A (about 95 kb) were found in all *P.s. glycinea* strains investigated.

In cor+ isolates, plasmids of classes B and D were mostly present and class C plasmids dominated in cor− strains. According to the plasmid patterns, the *P.s. glycinea* isolates were divided into several groups (ULLRICH et al., 1991). When strains isolated from the same field but from different seasons were compared, a conservation of plasmid pattern was observed. Plasmid profiles of 28 strains of the GSPB-collection did not correspond to their race.

Copper resistance of P.s. glycinea strains

For growth in the presence of copper, the cultures were grown for 3 d at 28 °C on standard I agar plates containing 2.0 mM copper sulfate. Among 47 *P.s. glycinea* isolates tested, the cor^+ strains grew well in contrast to all cor^- strains but one.

Race determination

In order to determine the race affiliation of the *P.s. glycinea* isolates, the strains were tested for elicitation of a compatible or incompatible reaction on eight differential soybean cultivars: Acme, Bicentennial, Chippewa, Flambeau, Harosoy, Merrit, Norchief, and Peking. References were type strains from the GSPB-collection. All cor^+ field isolates tested belonged to race 4 in contrast to the cor^- strains which could be grouped into race 9 or into an undefined race which was not covered in the reaction scheme of the known races of *P.s. glycinea*.

Homology of P.s. glycinea plasmid DNA to coronatine specific genes

Plasmids were isolated from various *P.s. glycinea* strains, and hybridized with a DNA fragment carrying genes involved in coronatine synthesis in *P.s. tomato* (BENDER *et al.* 1991). When non-digested plasmid DNA was probed with pSAY1, only the largest plasmid band of approximately 95 - 100 kb of each cor^+ strain hybridized to the probe whereas no hybridization signal could be detected in either plasmids or chromosomal DNA of cor^- strains. When pSAY1 was probed to *SstI*-digested plasmid preparations of various strains from the field-isolations or the GSPB, hybridization occurred to six typical *SstI* fragments similar in molecular size in all cor^+ strains. In contrast, no homology to DNA fragments from plasmid DNA of cor^- strains was observed. Furthermore, plasmid DNA of several *P.s. glycinea* isolates was digested with *Bam*HI and probed with pSAY1. Besides four fragments which were common in all cor^+ strains and also typical for pSAY1, two additional *Bam*HI-fragments hybridizing with pSAY1 were observed. Sizes of these additional bands varied among different strains and revealed RFLP independent from the race (Fig. 2).

PCR-analysis of cor^+ and cor^- P.s. glycinea strains

The *Tn5*-derived sequence "CAGGACGCTACTTG" was used as primer to perform PCR directed fingerprinting of genomic DNA of coronatine-producing and non-producing *P.s. glycinea* strains (ULLRICH *et al.*, 1992). PCR was carried out with genomic DNA of 9 cor^+ and 9 cor^- *P.s. glycinea* field-isolates. Four distinct amplification products similar in cor^+ and cor^{--} strains were seen.

However, the cor^+ isolates produced an additional signal at 5 kb, which was not found for cor^-- strains. This enabled us to differentiate both toxin-related phenotypes.

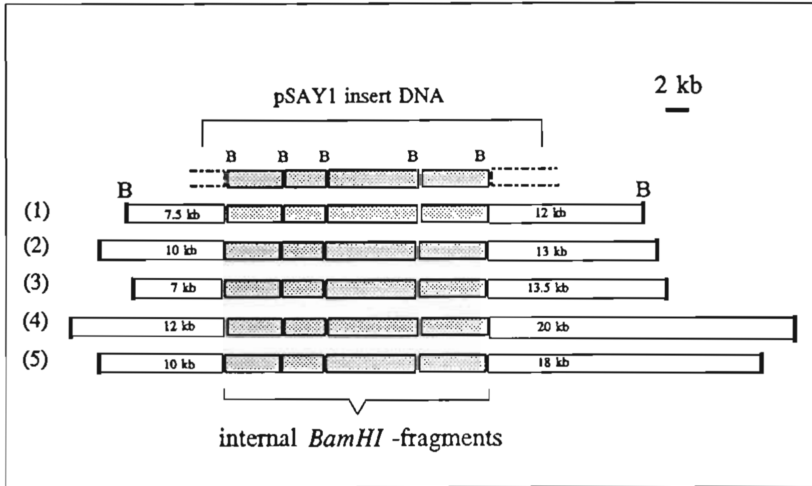

Fig. 2. A scheme for restriction fragment length polymorphisms in *Bam*HI-digested plasmid DNA of various *P.s. glycinea* strains hybridized to pSAY1. (1) - (5): different coronatine-producing strains from the field isolation and the GSPB. B: *Bam*HI-restriction site.

DISCUSSION

A race-specific correlation of coronatine synthesis and copper resistance has been found among 47 *P.s. glycinea* strains tested. Both properties had been linked to plasmids in *P.s. tomato* (BENDER et al., 1989; BENDER & COOKSEY, 1987). Thus, copper resistance is a new criterion for differentiation within pv. *glycinea*. This study revealed a diversity of plasmid patterns in *P.s. glycinea* from various geographic origins. Similar results were obtained in *P.s. tomato* (DENNY, 1988). DNA hybridization of *P.s. glycinea* plasmids with a DNA fragment involved in coronatine synthesis confirmed a strong conservation of biosynthetic genes in plasmid DNA of cor^+ isolates. However, restriction analysis of plasmid DNA homologous to pSAY1 manifested that the conserved coronatine-biosynthesis region in *P.s. glycinea* is flanked by non-conserved DNA seen as an RFLP. These results are consistent with the findings of YOUNG *et al.* (1992) who by saturation mutagenesis limited the DNA region of

P.s. glycinea essential for coronatine synthesis to 27 kb. None of the cor⁻ strains possessed DNA homologous to pSAY1 which can thus be used for detection of coronatine producing *P.s. glycinea* isolates. The classification of both types of strains into pathovar *glycinea* was not only achieved by pathogenicity tests but also by PCR-analysis with a random primer. Other pathovars of *P. syringae* gave rise to different PCR signals (S. BERESWILL & K. GEIDER, unpublished). Comparable results were obtained for aggressive and non-aggressive strains of the phytopathogenic fungus *Leptosphaeria masculans* (SCHÄFER & WÖSTEMEYER, 1992).

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An explanation for the symptomless "immune" response of tobacco leaves inoculated with incompatible bacteria

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The hypersensitive reaction (HR) of plants is a widely used assay for quick demonstration of bacterial pathogenicity (KLEMENT, 1963). The HR test is also used for the selection of *hrp* negative or avirulent mutants of plant pathogenic bacteria in genetic experiments. The HR is correlated with a rapid tissue necrosis in incompatible plants. However, visible necrosis of the inoculated leaf tissue is not always apparent and the plants sometimes remain symptomless. It is still unknown which defence mechanism is responsible for the symptomless "immune" response.

It was found repeatedly that *Pseudomonas syringae* pv. *phaseolicola* caused a necrotic HR in green tobacco leaves below 26°C, whereas the plants remained symptomless above 26°C. In contrast, *Pseudomonas syringae* pv. *pisi* induced the HR also at higher temperatures and in senescent leaves pv. *phaseolicola* induced HR also above 26°C. Why the leaves remained symptomless towards pv. *phaseolicola* at higher temperatures is not understood. However, these experiments indicated that the higher temperature did not *perse* inhibit development of the HR, because both pv. *pisi* (in green leaves) and pv. *phaseolicola* (in senescent leaves) induced the HR at temperatures above 26°C. When the multiplication of pv. *phaseolicola* was compared *in vitro* and *in planta* at 20 and 30°C, we found that the generation time *in vitro* was nearly the same at both temperatures : 2 hrs 45 min at 20°C and 2 hrs 30 min at 30°C. The bacterial population in tobacco leaves decreased during the first 8 hrs, after which a constant level was reached at both temperatures (Fig. 1). The fact that pv. *phaseolicola* multiplied *in vitro* at 30°C but did not cause HR at 30°C indicated that inhibition of bacterial growth should be attributed to another host response than HR at the higher temperature.

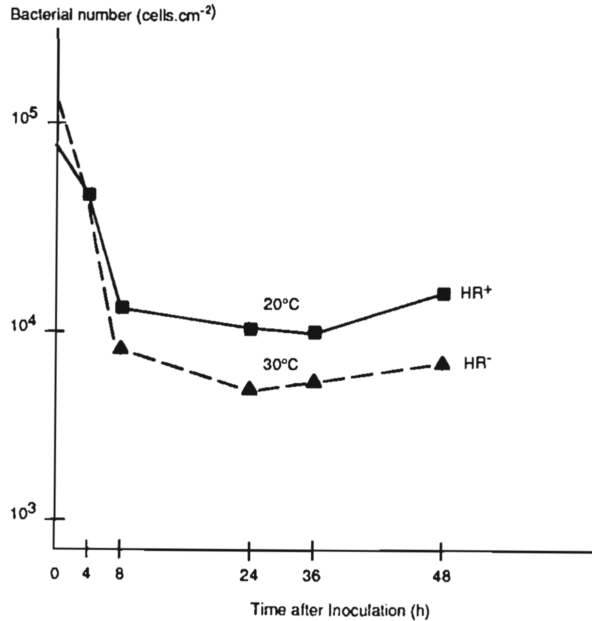

Fig. 1 : Population of *P. syringae* pv. *phaseolicola* (strain GSPB n° 656) in White Burley tobacco leaves at 20°C or 30°C

Recently we have shown that the HR is not the only host response of tobacco leaves in the early period after bacterial inoculation. At least two general defence mechanisms exist parallely in leaves, and they are triggered by the same pathogen.

One is the widely investigated HR and the other is the less known early induced resistance (EIR) (BURGYÁN and KLEMENT, 1979). How these two defence mechanisms influence each other was never investigated. We concluded from the experiments presented here that usually the HR is induced before the EIR develops. Thus the hypersensitive necrosis can appear (Fig. 2a) however, in certain cases the EIR develops very early by inhibiting initial bacterial growth which is necessary for triggering the HR. As a consequence the inoculated plant remains symptomless (Fig. 2b).

Because these two host responses overlap each other it is important to discuss the defence mechanisms in detail. First of all we would like to stress the importance of the early period during the development of the HR. As it was established before, there are 4 distinguishable periods during the development of the HR, these are : induction time, latent period, plant cell or tissue collapse, inhibition of bacterial growth (KLEMENT, 1982). The induction time is the most

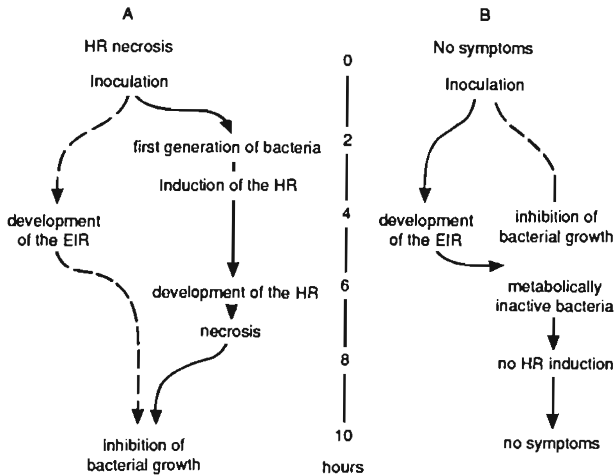

Fig. 2 : Parallely developing early host responsive of tobacco leaves triggered by *P. syringae* pv. *phaseolicola*.

a) hypersensitive reaction (HR) is induced before the early induced resistance (EIR) develops

b) EIR develops in a very early period after inoculation inhibiting initial bacterial growth which is necessary for triggering the HR. Consequently the plant remains symptomless.

important period. If the pathogen is metabolically inhibited during this period, the HR can not be induced and the inoculated plant remains symptomless (DURBIN and KLEMENT, 1977).

Very decisive is the length of the induction time. It depends on the generation time of the bacterial strain and on environmental factors such as growth conditions of bacteria and plants. Therefore, the time necessary for the development of early induced resistance (EIR) has to be compared with the induction time of the HR. It was established earlier that the EIR usually develops within 3-6 hrs after inoculation (BURGIÁN and KLEMENT, 1979). In the case of pv. *phaseolicola* the induction time of the HR (2.5-3 hrs) is similar to the time required for development of the EIR. For this reason it was thought that the higher temperature has an influence both on the induction time of the HR and on the time required for development of the EIR. The induction times of pvs. *phaseolicola* and *pisi* were compared at 20°C and 30°C. These investigations showed that the induction time of pv. *phaseolicola* was longer (2.5-3 hrs) than that of pv. *pisi* (1.5-2 hrs) in tobacco leaves. However, the higher temperature (30°C) did not influence the induction time of HR of pv. *phaseolicola*. Therefore, we determined the time required for the

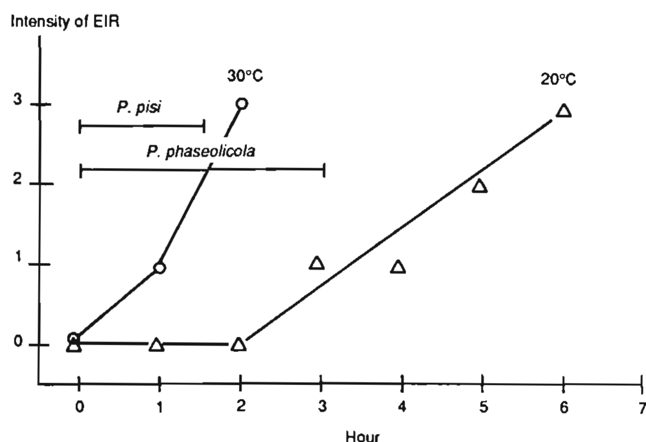

Fig. 3 : Development of the "early induced resistance" (EIR) in tobacco leaves at 20°C or 30°C compared with the HR induction time of *P. pisi* and *P. phaseolicola*, as indicated by the horizontal bars.

development of EIR at 20°C and 30°C. The development of the EIR was determined by the inhibition of the HR. Because pv. *phaseolicola* can not induce HR at 30°C, we had to measure the development time of the EIR in an other system. In this system the inducer of EIR was *P. fluorescens* and the challenger of the HR was pv. *pisi* (BURGIÁN and KLEMENT, 1979). The results are shown in Fig. 3.

We found that the time for the development of the EIR was significantly shorter at higher temperatures. At 30°C only 1-2 hrs but at 20°C 5-6 hrs long periods were required for the development of the EIR. Since it was already proved that the HR induction time of pv. *phaseolicola* is 2.5-3 hrs, these data suggest that the quicker development of the EIR (1-2 hrs) at 30°C may inhibit the multiplication of pv. *phaseolicola* during the induction time of the HR. The consequence of the inhibition of bacterial growth is manifested by the absence of the hypersensitive necrosis, since the HR can be induced only by metabolically active bacterial cells. This effect does not exist in the case of pv. *pisi*, because the induction time of the HR is shorter (1.5-2 hrs), so that the HR is induced before the EIR is fully expressed and effective.

Our experiments also showed that the EIR developed earlier in young leaves than in old and weakly senescent leaves. For this reason the HR induced by pv. *phaseolicola* can appear in senescent tobacco leaves but not in young green leaves at 30°C.

According to earlier results the EIR can be prevented by inhibition of protein synthesis, such as heat-shock or by pre-treatment of leaves with actinomycin D

(HEVESI *et al.*, 1981). Instead of the "symptomless response" the HR appeared when the tobacco leaves were pre-treated by heat-shock (at 50°C for 13 sec.) before inoculation with pv. *phaseolicola* and incubation at 30°C.

Summarizing our experiments we conclude that during the early period after inoculation at least two host responses exist parallely, the HR and the EIR. In certain cases may develop earlier, before the induction of the HR, so that the plants remain symptomless.

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Levansucrase as a possible factor of virulence? Characterization of the enzyme and occurrence in the *Pseudomonas syringae* group

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1. Abstract

Levansucrase of *Pseudomonas phaseolicola* is a small, stable enzyme, which is well adapted to a hostile environment. As judged from its kinetics, it may well be active in the intercellular space. Composition of the synthesized levan is not homogenous but depends on environmental conditions. The enzyme can act as a levanase, when sucrose is no longer available in heavily infected leaves. Levansucrases from different organisms are similar in molecular weight. These enzymes are produced constitutively by all of the *P. syringae* pvs. investigated, but are inducible in *P. fluorescens*. This suggests that the enzyme plays a role in pathogenesis.

2. Introduction

Pseudomonas syringae pv. *phaseolicola* is the agent of halo blight of bean. When the typical water-soaked spots appear, the bacterial extracellular polysaccharides (EPS) levan and alginate are detectable in the infected leaf. The EPS fills the intercellular space and covers the bacteria. Both types of EPS are produced *in planta* as well as *in vitro* when the bacteria are grown with sucrose as the only carbon source. For the synthesis of levan the pathogen possesses an exo-enzyme, levansucrase. Levansucrase splits sucrose and polymerizes the fructose residues to levan (ß-2,6-fructan) whithout any cost of energy while glucose is released. The enzyme is produced by several plant pathogenic bacteria constitutively (Sauerstein and Reuter, 1988; Gross et al., 1987; Gross et al.,

1990). Besides these, levansucrases from non-pathogenic bacteria are well known (Loitsanskaya et al., 1971; Elisashvili, 1980; Lyness & Doelle, 1983; Tkachenko & Sergeeva, 1990; Takahama et al., 1991), but in these bacteria production is regulated by substrate concentration. Constitutive synthesis and excretion of levansucrase in plant pathogenic bacteria may have evolved as an adaptation to survival in the intercellular spaces of plants, where sucrose is available in sufficient amounts. Organisms that withdraw assimilates from the plant by levansucrase have several advantages, for instance, production of a levan layer immediately after invasion of the plant tissue protects the parasite from recognition and at the same time may weaken the host by deprivation of sucrose. Furthermore, a carbon source (fructose) is accumulated and stored, which may be degraded later on by the pathogen when the resources of the host are exhausted. To assess whether the properties of levansucrase allow the proposed mode of action during pathogenesis, the enzyme from *Pseudomonas phaseolicola* was purified to homogeneity and characterized. In addition, 18 isolates from other *Pseudomonas syringae* pathovars were screened for production of levansucrase. The molecular weights of the enzymes were compared with that of levansucrase from *Pseudomonas phaseolicola*.

3. Purification and characterization of levansucrase from *Pseudomonas phaseolicola*

Supernatants of a 36-hour-old culture from mineral medium containing 1.2% sucrose as the only carbon source were harvested after EDTA-treatment (10 mM). The supernatant was filter-sterilized and immediately fractionated by anion exchange chromatography, followed by hydrophobic interaction chromatography. At this stage the enzyme eluted as a single symmetric peak. The eluate was concentrated by ultrafiltration if necessary. A typical elution profile is shown in figure 1.

The molecular weight of the purified enzyme was estimated by SDS-PAGE and native gradient gel electrophoresis. In SDS-PAGE the reduced, denatured protein exhibited a molecular weight of 45 kD, in native PAGE it was 80 kD. These properties are comparable to those of several known levansucrases (Cote, 1989; Gross et al., 1992). The Michaelis constant for sucrose hydrolysis to glucose and fructose was 0.16 M sucrose. Saturation of cleavage as well as synthesis took place at 0.3 M sucrose. The product glucose is a efficient inhibitor: even 1 mM glucose inhibited the reaction, 30 mM stopped the reaction completely. Because glucose is an easy-to-use carbon source for these bacteria, product inhibition is

not likely to occur *in planta* or, seen from the other side, glucose concentration can regulate the activity of the enzyme which otherwise would act autonomously in the intercellular space.

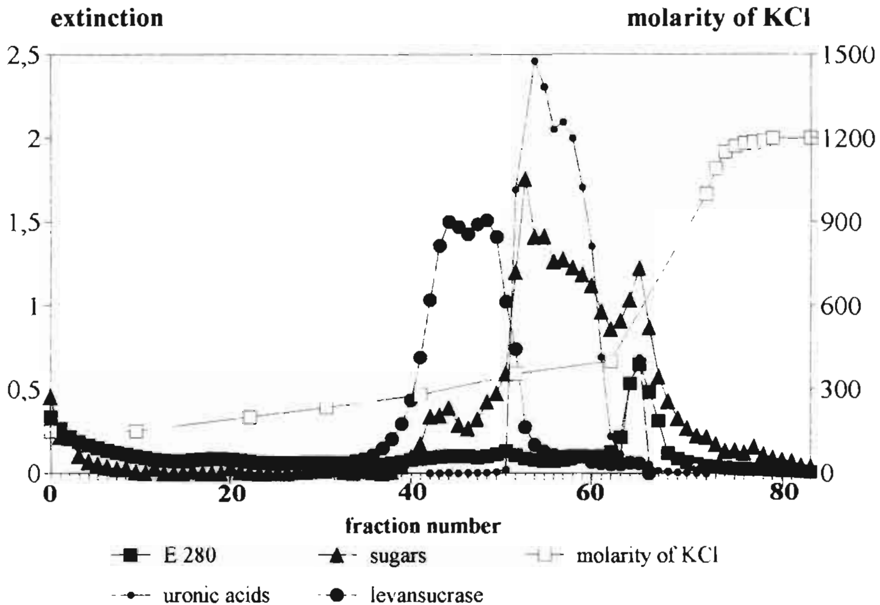


Figure 1: Anion exchange chromatography of supernatant from a 36-hour old culture of *Pseudomonas phaseolicola* on a TMAE-Fraktogel. The salt gradient was from 150 to 400 mM KCl. Levansucrase eluted at about 280 mM KCl. The enzyme was detected by activity.

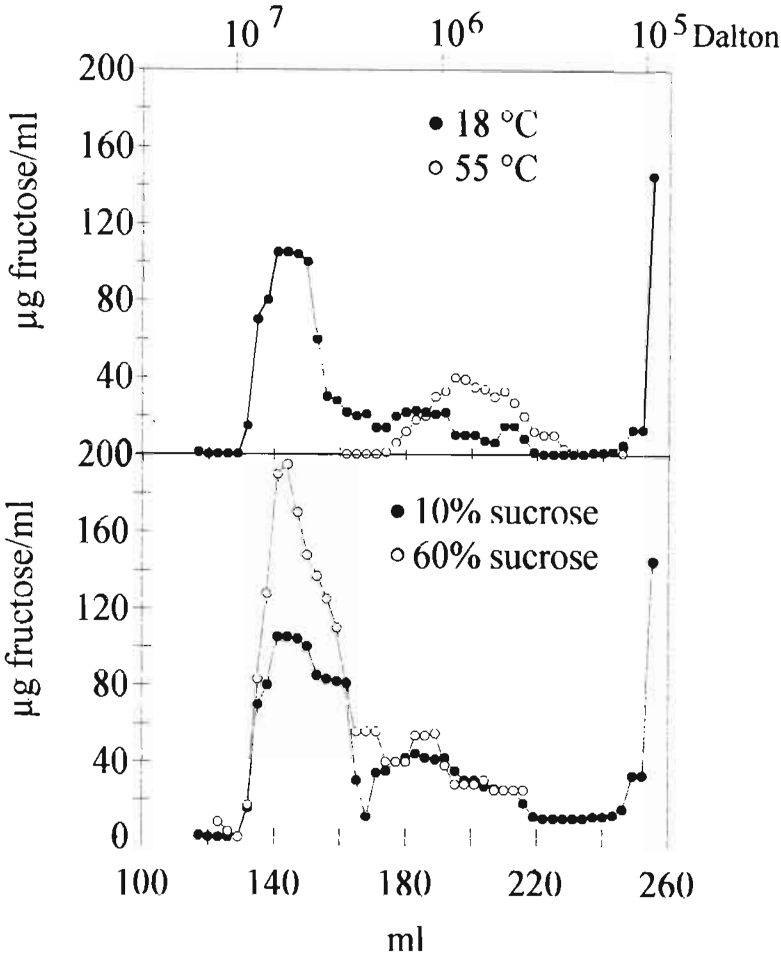
The enzyme tolerates heat up to 60°C (though levan production is maximal at about 18°C), pH-shifts (broad optimum: pH 5.5 to 7.5), reducing agents in high concentration (indicating the absence of disulphide bridges), detergents (active in 0.1 % SDS), protease activity, and repeated freezing-thawing (Hettwer et al., 1991).

These features describe the levansucrase as a small stable protein which is well suited to act under different, even unfavourable, conditions.

4. Relation between enzyme and product

Quantity and quality of levan produced under different temperature and sucrose regimes were compared by gel permeation chromatography and a ketose-specific assay (anthrone-test). At high temperature (37 or 50°C), a much smaller amount of levan was produced, with a significantly smaller molecular weight than that produced at 18°C (fig. 2). More levan was produced in low sucrose concentrations

(10%) than in high concentrations (60%), but the amount of high-molecular-weight levan was less in 10% sucrose than in 60% sucrose (fig. 3).
Because the enzyme should act autonomously in the intercellular space, the type of levan produced will depend directly on external, variable conditions such as temperature and sucrose concentration. These are the main variable parameters in leaves because they depend on diurnal rhythms. Whether variations in levan composition influence the progress and establishment of infection is an open question.



Figures 2, 3: Gel permeation chromatography of levan produced under different temperatures (2) and sucrose concentrations (3), respectively.

Besides the presumed role of levansucrase in early stages of infection to produce a "coat" of levan over the pathogen, a second ability may be important in later

stages. Levansucrase is able to split levan, to supply a new carbon source when the first substrate, sucrose, is exhausted.

Figures 4 and 5 clearly demonstrate the action of levansucrase on pure levan. Without addition of levansucrase there is only little ketose-positive material smaller than 10^5 Dalton, but after incubation with the enzyme, a lot of smaller molecules are detectable.

These diagrams also show that levansucrase from *Pseudomonas phaseolicola* binds to levan from *Erwinia amylovora*. This means that there is no specifity for enzyme and product from different organisms.

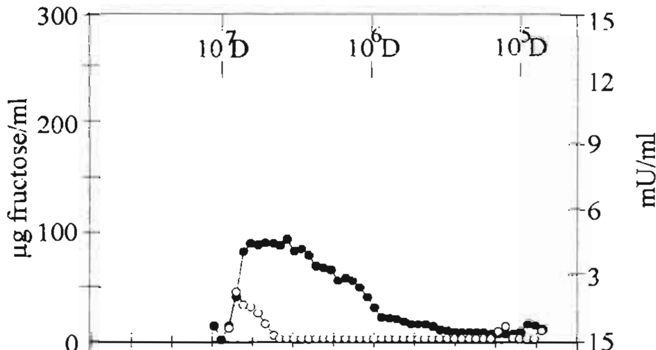


Figure 4

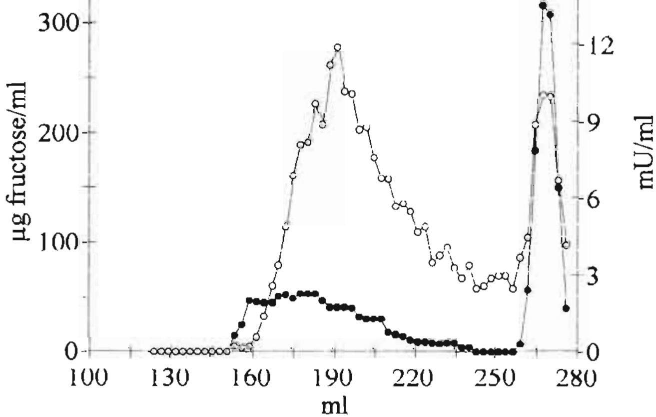


Figure 5

Figures 4, 5: Gel permeation chromatography of levan from *Erwinia amylovora* (4), and of levan from *Erwinia amylovora* incubated with levansucrase from *Pseudomonas phaseolicola* (5), (●) µg fructose/ml, (o) activity.

5. Comparison of several *Pseudomonas syringae* pathovars

To investigate whether different types of levansucrases are produced by different pseudomonads twenty strains of several *P. syringae* pathovars and one strain of *P. fluorescens* (levan-positive biovar), *P. viridiflava* and *P. cichorii* (as negative

control) were cultured under conditions normally used for *Pseudomonas phaseolicola*. Culture supernatants were obtained and partially purified by anion exchange chromatography. All samples were compared by gel electrophoresis (SDS-PAGE and native PAGE). By gel electrophoresis all of the levansucrases appeared to be of similar molecular weight. Levansucrase was detected within the gels by activity staining. Upon incubation in sucrose buffer, a slight turbidity marks the position of levansucrase (Pabst et al., 1979). The comparison of the isolates was difficult because more than one band of activity was often seen (perhaps due to capture of membrane-associated forms during purification). Clearly distinct were the bands in lane 4 (*P.s. pisi*) and lane 11-14 (*P.s. atrofaciens* V20, three isolates of *P.s. coriandricola**). These isolates possess levansucrases of higher molecular weight. Further studies on the relationship of these levansucrases are in progress.

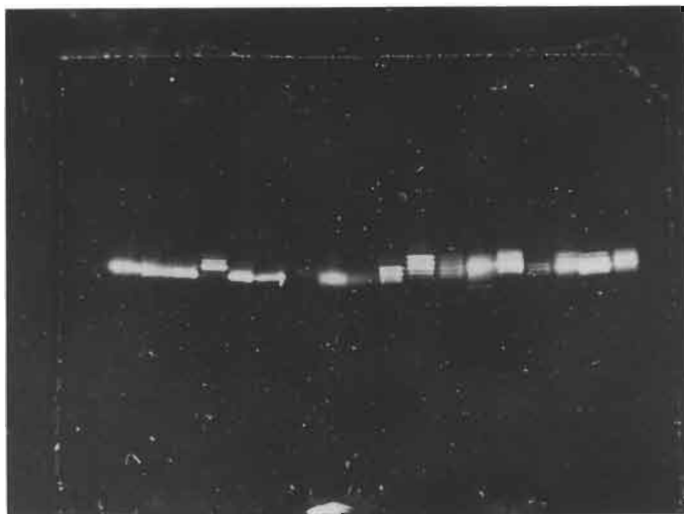


Figure 6: Native gradient gel electrophoresis (5-25%) of partially purified levansucrases from *P. fluorescens* and 17 strains of different pvs. of *P.syringae*. From left to right: *P.s. tomato* (Ontario, GSPB 487), *P.s. lachrymans* (83), *P.s. pisi* (537a2), *P.s. aptata* (Z 84-1), *P.s. tabaci* (DSM 50312), *P. fluorescens* (G-1), *P.s. glycinea* (2038, 9073), *P.s. atrofaciens* (NCPPB 2612, V 20), *P.s. coriandricola** (W 43, Cor 31, Cor 10), *P.s. syringae* (NCPPB 2842, R 32), *P.s. phaseolicola* (Kl S1, 1321). For every sample the same amount of activity was used. *Name not yet accepted.

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Difference of *Xanthomonas campestris* pathovars (*vesicatoria, glycines, phaseoli*) in pathogenicity and bacteriological properties on host and non-host plants

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INTRODUCTION

Bacterial strains belonging to the species *Xanthomonas campestris* (*Xc*) are difficult to identify because of their similarity in morphological, biological and physiological properties (13). At present, strains of *Xc* are distributed among pathovars depending upon their pathogenicity to various plants (2, 6, 14).

Phytopathogenic specialization within the genus *Xanthomonas*, although poorly understood, is of fundamental importance to modern agriculture since representative pathovars cause one or more diseases of practically all major groups of higher plants (12). Many *Xanthomonas* are highly adapted to causing diseases on define host-plant species, though there are reports of their multiplication and isolation from alternate hosts (3, 4, 7, 10, 11, 12).

Many plant diseases are caused primarily by various pathovars of *Xanthomonas campestris* in Bulgaria. Bacterial spot of tomato, bacterial pustule of soybean and common bacterial blight of bean are of great economic importance for our country.

In this paper we have explored aspects of host range in various pathovars (host or non-host combinations). We describe symptom development on different host and non-host plant species upon inoculation by pv. *vesicatoria, phaseoli* and *glycines*.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains were isolated from pustule infected soybean leaf, common blight infected bean leaf and bacterial spot infected tomato fruits collected from Bulgaria. Bacterial strains were identified as *Xanthomonas campestris* pv. *glycines* (*Xcgl*), pv. *phaseoli* (*Xcph*), pv. vesicatoria (*Xcv*) (collection of Plant Protection Institute, Kostinbrod, Bulgaria).

The natural bacterial strains of XcvPT 58 and 67 were isolated from dry spots on the fruits sort "Drouzhba" and "Ideal" and they are related to pepper tomato pathotype. XcvT 42 was isolated from watersoaked spots and was relating to tomato pathotype.

Inoculation

Cross inoculation tests were done on the following hosts and non-hosts : *Soya glycines*, *Phaseolus vulgaris*, *Lycopersicon esculentum*, *Pisum sativum*, *Cucumis sativa* and *Capsicum annum*. Primary and trifoliated leaf of 3 week old healthy soybean (S 1346), bean (Galji) and several fully expanded complete leaf of healthy tomato (Ideal), cucumber, pepper (Kyrtovska) and peas were inoculated by the vacuum infiltration method (1) and they were infiltrated into these plant species at a concentration of 10^8 cfu/ml. The inoculated plants (20) were kept in the greenhouse (20-26°C). All bacterial strains were grown for 36 hours at 28°C on PSA. Cells were suspended in sterile distilled water. Watersoaking and/or necrotic lesions development on leaf or stems were considered evidence for pathogenicity.

The inoculation was done using the following scheme with the bacterial strains *XcvT* 42, *XcvPT* 58, 67 ; *Xcgl* 19, 27 ; *Xcph* T13 : 1. host - reisolation (R) host - 4th generation R : 2. non-host - R - non-host till 4th generation R : 3. non-host - R - host - R - non-host till 4th generation R.

Reisolations were done from single typical spot on the leaf on the host and non-host by the standard bacteriological methods (9).

Bacterial characterization

Bacteriological tests were performed according to the determinative schemes described by DYE (5), BRADBURY (2) and SCHAAD (9). Xanthan synthesis was determined by the method of JEANES *et al* (8). Pathogenicity of the reisolates were tested by : 1. HR on tobacco (9) : 2. vacuum infiltration to the host.

RESULTS AND DISCUSSION

Pathogenicity and symptomathology of bacterial strains

Pathogenicity and symptomathological variation was clearly shown among pv. of *X. campestris*.

All strain isolates and reisolates from tomato (*Xcv* - 42, 58, 67) caused symptoms characteristic for bacterial spot on tomato leaf (Ideal). Four days after the infiltration dark brown spots were formed. Later around them joining yellow zones were formed.

Xcv was not pathogenic on cucumber and peas. *XcvT* 42 induced the disease on bean and soybean (on all reisolates to 4th generation) and *Xcv* 58 on pepper and bean, but not on the soybean leaf. *XcvPT* 68 was found to be pathogenic on pepper and soybean (to 4th generation).

On the soybean leaf *XcvT* 42 and *XcvPT* 68 strains formed small yellowish limited between the neurature on the 2 to 3 days after the infiltration. The tissue among the spots is chlorotic around them on the 5-6 day a yellow-orane stripe was formed. The spot type resembled the symptoms of the bacterial blight on bean.

On the bean leaf isolates *XcvT* 42 and *XcvPT* 68, irregular watersoaked spots, were formed 36 hours after the infiltration. The tissue gradually became necrotic and the spots dark-brown. The leave surface eventually turned yellow.

XcvPT 58 and 67 strains formed single round brown spots with a light center on the pepper leaf 4 to 5 days after inoculation.

The soybean isolates *Xcgl* 19 and 27 did not induce disease on cucumber, peas and pepper. Both isolates and their reisolates (4th generation) incited typical symptoms for the pustule bacteriose on the main soybean leaf (S 1346). Both isolates differed with respect to their pathogenicity on bean. *Xcv* 19 did not induce disease on bean, while isolate 27 and its reisolates (4th generation) was strong pathogenic on bean. Symptoms appeared 24 hours after infiltration as big irregular watersoaked spots.

The symptoms on the tomato leaf did not differ (both isolates and reisolates). The spots were small, non transparent and the tissues around were chlorotic. Single spots were detected on the stems too.

Xcph T13 did not incite the disease on cucumber, peas and pepper. On the bean leaf the spots were typical for common blight. Thirty-six hours after the bacteria infiltration, the isolation on the soybean leaf spots characteristic to the pustule bacteriose were formed.. The spots on the tomato leaf were small brown, without zone.

All reisolates of *Xcv*, *Xcph*, *Xcgl* from host and non-host plants induced the disease on the main host and the symptoms did not differ from those of the disease. They all gave HR from 24 to 48 hours after injection to tobacco leaf.

Characterization

The identification of all isolates and reisolates (4th generation) using the classical scheme showed that the basic property characteristic for *Xc* group were conserved. Some differences were registered on some reisolates from 3rd and 4th generation (*XcvT* 42 3-4 generation isolate on soybean and bean ; *Xcgl* 19 3-4 generation reisolate on tomato). They concerned acid production from : mannose, D ribose, xylose, L arabinose, D arabinose, lactose, raffinose, D cellobise and D+ melibiose.

The reisolates inhibited pectolytic activity from *Xcgl* on bean and from *Xcgl* 19 (3-4 generation reisolate), as well as all reisolates of *Xcph* T13 no matter of the host. The initial strains had not pectolytic activity.

No remarkable difference in the chemical formula of Xanthan produced by the various pvs (isolates and reisolates) in host and non-host plants was observed. Results of these studies will be published elsewhere.

From the studies carried out it can be concluded that host specificity of *Xanthomonas* pv. *vesicatoria*, *glycines* and *phaseoli* is not a stable character.

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Towards the characterization of early bacterial functions in *Erwinia amylovora*-host plant interactions

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Abstract

As far as aerial pathogens are concerned, many pathogenicity tests are done by wounding the plant or plant tissues. Such methods are often used for screening mutants in order to define the functions necessary for bacterial virulence. They, however, fail to isolate mutants which would be altered in the very early steps of the infection process like, for instance, bacterial penetration into the host.

Assuming that the first events of the interaction between a pathogen and its host involve surface components, we screened a mutant library of *Erwinia amylovora* for membrane alterations. Stable insertional mutants were obtained from *E. amylovora* strain CFBP1430, using the phage Mu derivative MudIIPR13 (BARNY *et al.*, 1990). Potential membrane mutants were selected on the basis of modified sensitivity to various agents known to interfere with outer membrane stability (sodium deoxycholate, EDTA, antibiotics). A preliminary analysis of these mutants is presented: their pathogenicity, however, has not been determined yet. On the other hand, membrane phenotypes that were observed among pathogenicity mutants are also described.

Keywords : *Erwinia amylovora*, fireblight, cell surface, pathogenicity, genetics.

Introduction

Erwinia amylovora causes fireblight, a necrotic disease of pome trees. Pathogenicity assays are usually performed by inoculating cut fruits, calli, leaves or stems with the bacterium. However, such methods fail to isolate mutants which would be altered in the very early steps of the interaction with the host plant.

Assuming that early events in the interaction involve bacterial surface components, we developed an approach based on the screening of transposon induced cell surface mutants. Potential membrane mutants were selected on the basis of modified sensitivity to various agents known to interfere with the outer membrane stability. Such phenotypes were also screened among our set of non-pathogenic mutants.

Selection of *E. amylovora* membrane mutants

Results

Insertional mutagenesis of *Erwinia amylovora* strain CFBP1430 was performed through infection by the phage Mu derivative MudIIPR13, giving 6,900 stable independent chloramphenicol resistant (Cm^r) clones (BARNY *et al.*, 1990). Each Cm^r clone was inoculated in L-medium (Miller's LB medium, except that NaCl was 5 $g.l^{-1}$, Miller 1972) supplemented with chloramphenicol (Cm, 10 $\mu g.ml^{-1}$) in a well of a 96-wells microtitration plate, grown overnight at 25°C and stored at – 80°C. For the screening of membrane mutants an aliquot from each plate-well was diluted about fifty times in 10^{-3} M $MgSO_4$. A drop (about 3 μl) from each well was deposited onto L- or M63-agar medium (Miller, 1972) supplemented with either sodium deoxycholate (DOC, 1 mM for L + DOC, 1.5 mM for M63 + DOC) or ethylene diamine tetraacetic acid (EDTA, 400 μM for L + EDTA, 20 μM for M63 + EDTA) or onto L-agar medium supplemented with ampicillin (3 $\mu g.ml^{-1}$), kanamycin (3 $\mu g.ml^{-1}$) or kasugamycin (100 $\mu g.ml^{-1}$). After a 2- (L media) or 3-days long (M63 media) incubation at 25°C, the growth of each clone on a given supplemented medium was compared to its growth on the same medium without any additive product. A mutant phenotype was attributed when the relative growth of the clone was different from the relative growth of CFBP1430 on similar media. Out of about 1,000 Cm^r clones, 50 prototrophic clones displayed a mutant phenotype. In order to determine the number of Mu DNA insertions in the genome of these mutants, Southern blot hybridization experiments were realized using a Mu DNA probe and *Bam*HI-digested DNA from the mutants. The 29 potential membrane mutants with a unique Mu insertion showed a large diversity of phenotypes. All these mutants previously proved to retain the ability to produce exudate when inoculated on apple calli (PAULIN & DURON, 1986). Therefore they had not been assayed for pathogenicity by inoculating pear or apple seedlings (BARNY *et*

al., 1990) and are thus different from the pathogenicity mutants mentioned below.

Prospective

Further characterization of these potential membrane mutants will include :

(i) Polyacrylamide gel electrophoresis of outer membrane proteins (OM-proteins) and lipopolysaccharide (LPS).

(ii) Determining the ability of membrane mutants to produce symptoms on host plants when inoculated either by wounding or by spraying seedlings.

(iii) Mapping of MudIIPR13 insertions :

- the first step is the search for insertions mapping in genomic regions previously shown to be involved in *E. amylovora* pathogenicity : the *hrp* region (for *h*ypersensitive *r*esponse and *p*athogenicity) previously described by several authors (BARNY *et al.*, 1990 ; BAUER & BEER, 1991 ; STEINBERGER & BEER, 1988 ; WALTERS *et al.*, 1990) in which we discriminate a subgroup of *d*isease *sp*ecific (*dsp*) functions ; and another genomic region, designed *ams* since it appeared concerned with amylovoran synthesis (MENGGAD, pers. comm. ; GEIDER, pers. comm.)
- the second step will be the mapping of MudIIPR13 insertions in the genome of the characterized cell surface mutants.

(iv) Identifying the product and function of any bacterial gene which might be involved in early interactions between *E. amylovora* and host plants as inferred from point (ii).

Membrane phenotypes among pathogenicity mutants

Out of 6,000 Cm^r clones, 53 pathogenicity mutants with a single MudIIPR13 insertion were obtained (BARNY *et al.*, 1990 ; THARAUD *et al.*, in preparation). These mutants were screened for sensitivity to DOC or EDTA either on L-agar medium or on M63-agar medium as described above. Eight clones exhibited a modified sensitivity when compared to CFBP1430. The results are presented in Table 1. Most insertions mapped

Strain	Aspect of the colonies on L-agar	Pathogenicity and *mapping*	Sensitivity to phage Ffm	LPS profile	Membrane phenotype			
					L + DOC	L + EDTA	M63 + DOC	M63 + EDTA
CFBP1430	mucoid (+)	+	Resistant (R)	+	+	+	+	+
PMV6028	slow growth rough	Dsp$^-$ *?*	Sensitive (S)	Modified (M)	as on L-agar	+++	very bad growth on M63	
PMV6041	rough acapsular	Dsp$^-$ *hrp-dsp*	S	M	+	+	–	–
PMV6047	+	Dsp$^-$ *hrp-dsp*	R	+	+	+	±	+
PMV6076	+	Hrp$^-$ *hrp-dsp*	R	not done (ND)	+	rough	–	–
PMV6089	rough acapsular	Dsp$^-$ *ams*	R	ND	rough	rough	+	–
PMV6101	+	Agr$^-$ *hrp-dsp ?*	S	ND	rough	rough	–	–
PMV6102	+	Hrp$^-$ *hrp-dsp*	S	ND	rough	rough	–	–
PMV6112	+	Dsp$^-$ *hrp-dsp*	S	ND	more mucoid	+	–	–

Tableau 1: Characteristics of *E. amylovora* pathogenicity mutants exhibiting membrane phenotypes

in the large *hrp-dsp* gene cluster above-mentioned. The pathogenicity phenotypes are indicated as follows : Agr$^-$, reduced virulence ; Dsp$^-$, no symptoms on host plants (Path$^-$), hypersensitive response on tobacco (HR$^+$) ; Hrp$^-$, HR$^-$Path$^-$. Five out of these 8 mutants could be infected by the phage Ffm, unlike CFBP1430. The virulent phage Ffm infects *E. coli* K12 and most rough mutants of *Salmonella typhimurium* or *Erwinia chrysanthemi* (WILKINSON *et al.*, 1972 ; SCHOONEJANS, pers. comm.). For the two latter bacterial species the phage Ffm appears to specifically attach to the core of LPS. Polyacrylamide gel electrophoresis of the LPS from some of the Ffm sensitive mutants were performed. The profiles observed after silver-staining proved to be similar to each other but different from the LPS profile of CFBP1430. The two distinct banding regions described by VANNESTE *et al.* (1990) were still visible. The low molecular weight region showing few large bands, which should correspond to the core part of the LPS, remained unchanged. The higher molecular weight heterogeneous region showing a scale of equidistant bands, which probably correspond to the O-antigen-like structure, appeared displaced towards lower molecular weights and presented a much lower number of bands. The LPS and OM-proteins profiles of all the mutants exhibiting both pathogenicity and membrane phenotypes will be studied.

Discussion

Bacterial cell surface components may play different roles in the bacterium-plant interaction. They could be directly involved in the interaction with a plant component. Alternatively they could be considered as structural components important for the membrane permeability (either selective or not). Some components might be specific receptors for signal molecules or nutrients.

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Electrolyte leakage from host and non host plants associated with *hrp* genes of necrogenic bacteria

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ABSTRACT

Necrogenic bacteria induce electrolyte leakage from tissues of host and non-host plants. A miniaturized technique easily allows the precise quantification of this process. Compatible and incompatible situations induced typical and distinct leakages of electrolytes, *i.e.* slow and progressive (disease reaction), or fast and intense efflux (hypersensitive reaction), respectively, in most tested interactions. In the interactions of *Erwinia amylovora* and *Ps. s.* pv. *syringae* with pear, the responses in compatible and incompatible situations were also distinct but different from the typical patterns, at least after the very first hours of interaction. When transposon pathogenicity mutants were used, it was found that mutants lacking functional *hrp* region were unable to induce any leakage from host and non-host plant tissue. Mutants affected in *dsp* region induced a weak leakage or no leakage at all, in both incompatible and compatible situations. In addition, this technique was used to assess the type of interaction between various plants and strains belonging to the ubiquitous *P. syringae* species. A host-specific interaction was shown for several pathovars and also for some strains within the *syringae* pathovar.

KEY-WORDS : Disease reaction, hypersensitive reaction, electrolyte leakage, pathogenicity mutants, *Erwinia amylovora*, *Pseudomonas syringae*

INTRODUCTION

During the interaction with plant tissues, necrogenic bacteria induce a leakage of electrolytes, leading either to hypersensitive reaction for incompatible situations or disease reaction for compatible ones. Monitoring of this leakage has been already described (ATKINSON & BAKER, 1987 ; BRISSET & PAULIN, 1991).

MATERIAL AND METHODS

We devised a miniaturized technique, shown on figure 1, which was used to analyse several bacteria/plant interactions (table 1).

	Strains			Plants	
Bacterial strains	wild type	*hrp*$^+$ *dsp*$^-$	*hrp*$^-$ *dsp*$^+$	compatible	incompatible
E. amylovora	1430	6069	6044	Pear	Tobacco
P. solanacearum	GMI 1000		GMI 1390	Tomato	Tobacco
X. c. pv. *vesicatoria*	GMI 8211		GMI 8212	Pepper ECW (susceptible)	Pepper ECW10R (resistant)
P. s. pv. *phaseolicola*	SD68			Bean	Pear
P. s. pv. *syringae*	DAN 86-4			Bean	Pear
P. s. pv. *syringae*	2027-37			Pear	Bean
P. s. pv. *tabaci*	2106			Tobacco	Pear, Bean
P. s. pv. *tomato*	2212			Tomato	Bean

Origin of transposon mutants :

- 6069-6044 INA-PG Paris (M.A. BARNY)
- GMI 1390 INRA-CNRS Toulouse (C. BOUCHER)
- GMI 8212 IGF Berlin (U. BONAS)

Table 1 - Characteristics of strains and type of interaction on plants

RESULTS - DISCUSSION

From every plant tested, with the exception of pear, bacteria induced a fast and intense leakage in incompatible situation (non host or resistant host cultivar), whether they induced a slow and progressive one in compatible situations. An example is given on figure 2. Inverse patterns were obtained on pear (fig. 3). In each case, we were able to differenciate a compatible from an incompatible situation on a given plant.

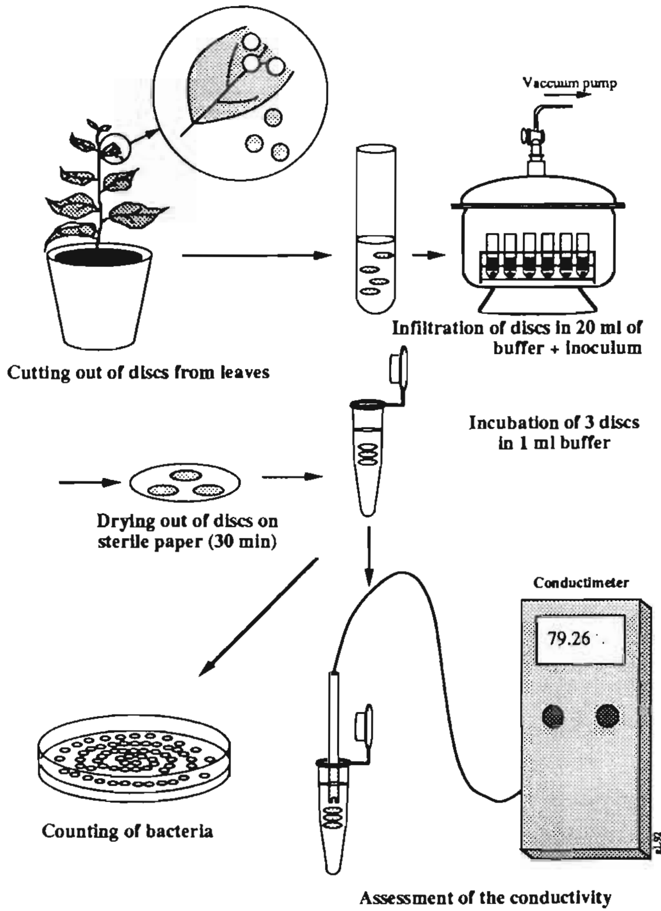

Figure 1. Micro method for the monitoring of electrolyte leakage in plant/bacteria interactions.

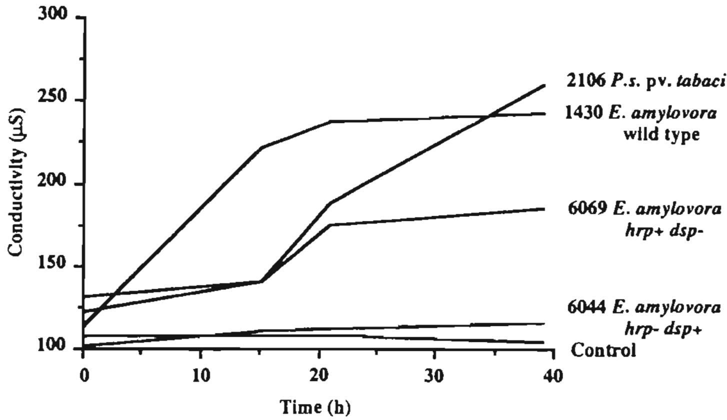

Figure 2. Electrolytes leakage induced on tobacco by *P. s.* pv. *tabaci* (compatible), and *E. amylovora* (incompatible) wild type and mutants.

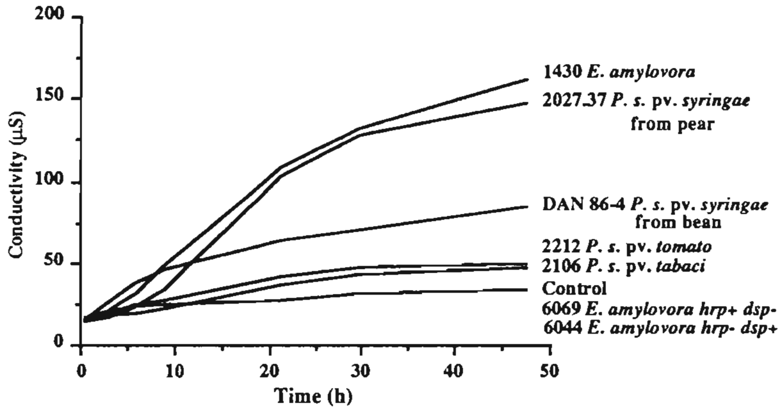

Figure 3. Electrolytes leakage induced on pear by wild types of : *P. s.* pv. *syringae*, *E. amylovora* (compatible), *P. s.* pv. *tomato*, pv. *tabaci* (incompatible), and by pathogenicity mutants of *E. amylovora*.

This result could be of particular interest for the characterization of pathovars and/or strains of the ubiquitous phytopathogenic bacterial species *Pseudomonas syringae* (fig. 3).

The ability of necrogenic bacteria to induce electrolyte leakage from host or non host tissues was correlated with a functional *hrp* (*h*ypersensitive *r*eaction *p*athogenicity) cluster since transposon *hrp*⁻ mutants never produced any efflux. Mutation in the *dsp* (*d*isease *sp*ecific) region reduced the ability of *E. amylovora* to cause leakage in incompatible situation (fig. 2), and suppressed the leakage in compatible situation.

CONCLUSION

The miniaturized method that we proposed provides a suitable and convenient tool to investigate the interactions between necrogenic bacteria and plants. A difference of patterns in the leakage of electrolytes in compatible and incompatible situations seems to be the rule. Therefore this technique allows the study of the expression and the role of the *hrp* and *dsp* genes in plant bacterial interaction : leakage is strictly linked to a functional *hrp* cluster for every tested bacteria. But, in the case of *E. amylovora, dsp* genes seem to be also involved even in the incompatible situation.

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Genetic characterization of a DNA region involved in amylovoran synthesis of *Erwinia amylovora* by complementation of *Erwinia stewartii cps* mutants

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ABSTRACT

A cosmid clone, containing a gene cluster required for production of the acidic exopolysaccharide (EPS) amylovoran by the fireblight pathogen *Erwinia amylovora*, was mutagenized with transposon Tn5 and selected mutations were crossed into the chromosome. Five complementation groups, designated *amsA - E*, were identified; all were essential for amylovoran synthesis and virulence in *E. amylovora*. The *ams* gene cluster was found to be related to the *cps* gene cluster for EPS synthesis by the corn pathogen *Erwinia stewartii*. Mucoidy and virulence were restored to mutants in four *E. stewartii* complementation groups by the cloned *E. amylovora ams* genes. Conversely, the *E. stewartii cps* gene cluster was able to complement mutations in *E. amylovora ams* genes. Correspondence between the complementation groups of these two species were determined. For characterization of EPS from *ams*- and *cps*- mutants with heterologous complementation, EPS-specific bacteriophages revealed evidence of an altered exopolysaccharide composition compared to the wild type EPS.

Keywords: exopolysaccharide, fireblight, pathogenicity, heterologous complementation

INTRODUCTION

Erwinia amylovora and *Erwinia stewartii* are related pathogens that cause fireblight on apple, pear and other rosaceous plants and Stewart's wilt on corn, respectively. Both bacteria produce large amounts of acid exopolysaccharide (EPS), which may function in protecting the bacteria against plant defense reactions (COPLIN & COOK, 1991). Furthermore, EPS causes wilting by occluding xylem elements. The biosynthesis and the chemical structure of the exopolysaccharides are not well understood. A preliminary structure of amylovoran has been proposed by SMITH et al. (1989). The basic sugars are glucuronic acid and galactose. *E. stewarti* EPS contains glucose in addition to these sugars (COPLIN & COOK, 1991). In this study, we found that cloned EPS genes from *E. amylovora* (*ams* = amylovoran synthesis) and *E. stewartii* (*cps* = capsular polysaccharides synthesis) will restore EPS synthesis to corresponding mutants of the other species. A DNA fragment from *E. amylovora* was found to carry a cluster of at least five complementation groups (or genes) required for amylovoran synthesis. In addition to the functional relationships between the biosynthetic genes, the regulation of EPS synthesis in these two species shares a common transcriptional activator, RcsA (BERNHARD et al., 1990).

RESULTS and DISCUSSION

Complementation of E. stewartii cps mutants with an E. amylovora genomic library.

The *cps* cluster in *E. stewartii* has been described by COPLIN & MAJERCZAK, (1990) and contains at least five *cps* complementation groups. A cosmid clone from an *E. amylovora* genomic library, constructed in vector pVK100, contained a 14.8 kb insert of chromosomal *E. amylovora* DNA. Plasmid pEA109 restored EPS-synthesis to *E. stewartii* strains DM144 (*cpsE*) and DM215 (*cps-192*) (Table 1), but not to a *cpsA* mutant. The *cps* mutants complemented by pEA109 produced less EPS than the *E. stewartii* wild type strain DC283, but they regained full virulence on sweet corn seedlings. The *ams* mutants were constructed by Tn5 mutagenesis of pEA109 followed by marker-exchange into the chromosome of a wild type strain. The *ams* transcriptional groups were defined by complementation tests between pEA109 mutants and subclones and chromosomal *E. amylovora* mutants (GEIDER et al., 1992) and by additional complementation tests between pEA109 subclones and *E. stewartii cps* mutants.

Table 1: Phage specificity and virulence for Erwinia strains and complemented mutants

strain	phage ϕ-K9*	phage PEa1(h)*	virulence**
E. stewartii:			
DC283	3	2	3
Gal8	0	0	0
DM144	0	0	0
DM144 (pEA109)	3	2	2.1
DM215	0	0	0
DM215 (pEA109)	0	2	2.3
E. amylovora:			
Ea1/79N	0	3	3
Ea1/79N (pES2144)	1	3	1 to 2
Ea1/79N-delAE	0	0	0
Ea1/79N-D4	0	0	0
Ea1/79N-D4 (pES2144)	2	2	0

*Ratings for phage sensitivity: 0, no reaction; 1, faint spot; 2, turbid spot; 3, clear lysis. Spots indicated degradation of EPS without cell lysis.

**Ratings for virulence: 0, no symptoms to 3: full symptoms. The virulence tests were performed on sweet corn seedlings using a watersoaking assay for *E. stewartii* strains and on slices of immature pears evaluating ooze production for *E. amylovora* strains. Symptoms were evaluated about 4 days after inoculation.

Functional correlations between E. amylovora ams- and the E. stewartii cps-regions.

A central fragment from pEA109 was sufficient to complement the *E. stewartii cps* region from *cps-192* to *cpsE* except *cpsC*. The *E. stewartii cpsC* region corresponded to *E. amylovora amsA* and the *cps-192* allele was functionally equivalent to *E. amylovora amsB*. Since DM220 *cpsD* was only complemented by subclones containing the *amsC-E* genes, it appears that these three genes may be one operon in *E. stewartii* (Fig. 1). The region of pEA109 responsible for complementation of *cpsE* has not been located. *E. amylovora ams*-mutants were likewise complemented by pES2144 (COPLIN & MAJERCZAK, 1990), carrying the *cpsA-E*

genes, but subclones of pES2144 failed to restore EPS-synthesis to any of them. Supplementation of an *E. amylovora* wildtype strain with plasmid pES2144 resulted in a decrease of its virulence on pear sclices (Table 1).

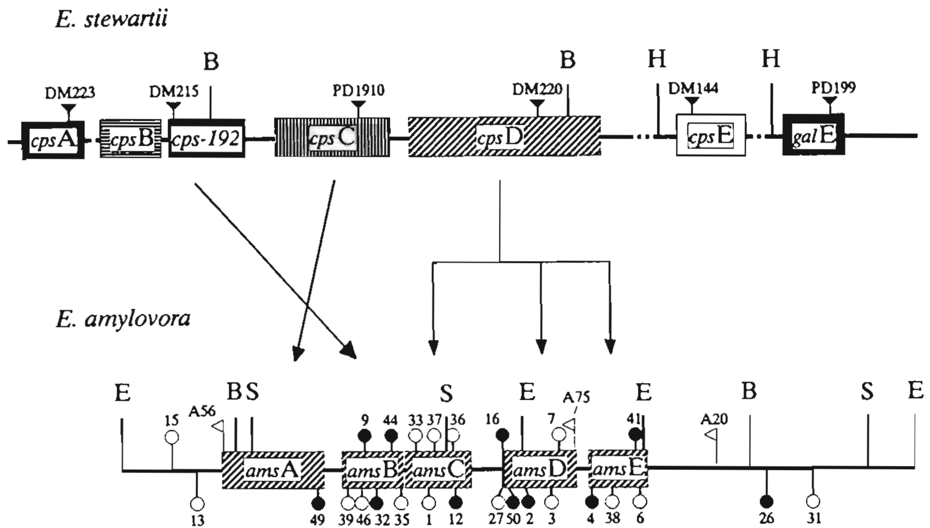

Fig.1: Comparison of *E. stewartii* and *E. amylovora* gene clusters involved in EPS-synthesis. Circles: Tn5 insertions in pEA109; filled circles and triangles: Chromosomal Tn5 insertions in *E. amylovora* and *E. stewartii*, respectively. Cleavage sites: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I.

Sensitivity of ams/cps merodiploids to EPS-specific bacteriophages.

Several EPS-specific bacteriophages were used to determine if complementation of *E. stewartii* and *E. amylovora* mutants by heterologous EPS genes might involve changes in EPS composition or structure. *E. amylovora* phage 4L (BILLING & GARRETT, 1980) degraded EPS of wild-type strains of both *E. amylovora* and *E. stewartii*, but did affect not acapsular mutants of these species. Similarily, sensitivity to phage PEa1(h) (HARTUNG et al., 1988) was restored by complementation of DM144 (*cpsE*) and DM215 (*cps-192*) with pEA109 and mutant Ea1/79N-D4 by pEA2144 (Table 1). These data suggest that phages 4L and PEa1(h) recognize a common feature of the two EPSs that is not altered by complementation with

heterologous EPS genes. On the other hand, a phage from *E. stewartii*, ϕ-K9 (BRADSHAW-ROUSE et al., 1981), appeared to be specific for *E. stewartii* EPS. ϕ-K9 only lysed wild-type strains of *E. stewartii* (Table 1). Complementation by pEA109 restored sensitivity of ϕ-K9 to DM144 but not to DM215. These findings suggest that *cpsE* and its corresponding gene on pEA109 encode common functions that do not change ϕ-K9 recognition, whereas complementation of *cps-192* by *amsB* probably results in altered EPS structure or composition. Both DM144 (pEA109) and DM215 (pEA109) regained water-soaking ability on sweet corn seedlings, indicating that any changes in the EPS structure on the later transconjugants did not affect virulence.

Complementation of the *E. amylovora amsE* mutant D4 with pES2144 restored EPS synthesis, but not virulence (Table 1). However, the transconjugants gained sensitivity to ϕ-K9. Considering the specificity of ϕ-K9, this result suggests that some *E. stewartii*-like EPS was produced by Ea1/79N-D4 (pES2144).

CONCLUSIONS

An *ams* gene cluster required for synthesis of the acidic exopolysaccharide amylovoran by the fireblight pathogen *E. amylovora* was cloned and characterized. The cloned *ams* genes could functionally complement *cps* mutants of *E. stewartii* and, conversely, cloned *cps* gnes could restore EPS synthesis to *E. amylovora ams* mutants. The *ams/cps* merodiploids were altered in sensitivity to phage ϕ-K9 specific for *E. stewartii* EPS indicating that they may produce altered EPS.

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Effect of induced protection on the expression of *hrp*-genes of *Erwinia amylovora* in tobacco leaves

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ABSTRACT

In tobacco leaves the hypersensitive response (HR) elicited by *Erwinia amylovora* was prevented 24h after intercellular injection with protein-lipopolysaccharides (pr-LPS, 250μg/ml) of *Pseudomonas syringae* pv. *aptata*. The effect of pr-LPS treatment on expression of *E. amylovora hrp* (hypersensitive response and pathogenicity) genes was tested *in planta* and *in vitro*. The *Escherichia coli* β-glucuronidase (Gus) gene was used as a chromosomal reporter gene to monitor the expression of two *hrp* loci of *E. amylovora*. The inhibition mechanism of the HR to *E. amylovora* by pr-LPS treatment is discussed.

INTRODUCTION

The pre-treatment of tobacco leaves with bacterial protein-lipopolysaccharides (pr-LPS), prevents the rapid hypersensitive resistance response against phytopathogenic bacteria and induces in the plant a state of protection that is favourable for both of the interacting cells: the plant cells do not die and the bacteria multiply in the intercellular space (MINARDI *et al.*, 1989). In the heterologous system *P. syringae* pv. *aptata*-tobacco leaves, the protection induced by the treatment of *P. syringae* pv. *aptata* pr-LPS has been extensively studied from the biochemical and physiological point of view (MINARDI 1991; VALENTI *et al.*, 1989). Although it is clear that the pr-LPS treatment alters the plant-bacteria recognition system by creating a state of tolerance between the two counterparts, the mechanism of induced protection is still poorly understood. Therefore we hypothesized that pr-LPS treatment could affect the expression of the so-called *hrp* genes, involved in the elicitation of the HR, in a way that the HR phenotype is suppressed.

To test this hypothesis we used the heterologous system *Erwinia amylovora*-tobacco leaves. *E. amylovora* is the causal agent of fire blight, one of the most devastating diseases affecting species of the subfamily *Pomoideae* including apple and

pear (ALDWINCKLE AND BEER, 1979). On nonhosts (such as tobacco) *E. amylovora* elicits the HR. The pre-treatment of tobacco leaves with pr-LPS of *P. syringae* pv. *aptata* induced protection against *E. amylovora*. The effect of pr-LPS treatment on the expression of *E. amylovora hrp* genes was tested in protected tobacco leaves and in a defined liquid *hrp*-gene inducing medium. The entire *hrp* gene cluster of *E. amylovora* had previously been cloned in the cosmid pCPP430, mutagenized with a transposon Tn*5* containing the *E. coli* β-glucuronidase (Gus) coding sequence, and marker-exchanged back into *E. amylovora* (BEER *et al.*, 1991). In this study, the Gus gene was used as a chromosomal reporter gene for monitoring the expression of two *hrp* loci of *E. amylovora*.

MATERIALS AND METHODS

In this study we used Ea321 mutants containing the same GUS insertions in the chromosome -Ea321::Gus73 and Ea321::Gus86, hrp$^-$- or in the plasmid -Ea321(p1055) and Ea321(p1020), hrp$^+$ -respectively. Those two *hrp* loci transcriptionally fused with Tn*5-gus*A1 are located in the highly conserved region between the identified *hrp* gene clusters of *Pseudomonas syringae* pathovars and *E. amylovora* (LABY and BEER, 1990). Moreover, they are particularly useful in regulation studies for their strong expression *in vitro* and in tobacco leaves (WEI *et al.*, 1992).

Small scale plasmid preparations were done by an alkaline procedure (BIRNBOIM, 1983). Bacterial transformation, preparation of competent cells and gel electrophoresis were carried out essentially according to MANIATIS *et al.* (1982). Strains of *E. amylovora* and *E. coli* were transformed by triparental mating according to the procedure of DITTA *et al.* (1980) or by electroporation (DOWER *et al.*, 1988). *E. coli* HB101 (pRK600) was used as a conjugative helper strain. Single-colony transfers were used to purify the transconjugants on selective media.

Pr-LPS were purified from *P. syringae* pv. *aptata* NCPPB2664 and injected (250 μg/ml) into tobacco leaves to induce protection against the wild-type strain of *E. amylovora* and its strains following the method previously described (MINARDI *et al.*, 1989).

β-glucuronidase (Gus) activity *in vitro* was assayed fluorometrically as described by JEFFERSON *et al.* (1987). The medium used for the Gus activity was an *hrp*-gene inducing medium (IM). Gus activity *in planta* was assayed as previously reported (WEI *et al.*, 1992).

RESULTS AND DISCUSSION

Treatment of tobacco leaves with pr-LPS prevented after 24h the hypersensitive confluent necrosis towards Ea321 and the hrp$^+$ mutants Ea321(p1055) and Ea321(p1020).

The pr-LPS treatment did not affect the growth of Ea321 or the mutants containing the same GUS insertions in the chromosome or in the plasmid.

In *hrp*-gene inducing medium amended with pr-LPS the Gus activity of hrp⁻ mutants was not significantly different from the control in which pr-LPS were not added to the medium. Thus pr-LPS alone did not have a nutritional effect that inhibited the *hrp* genes expression as it is found with rich media.

The physiological changes induced in tobacco leaves following the pr-LPS treatment might have a direct effect on the expression of *hrp* genes perhaps eliciting a supressor. To test this hypothesis, we measured the GUS activity of the bacteria after incubation *in planta*. Protected and unprotected tobacco leaves were challenged with the hrp⁻ mutants of Ea321 containing the *hrp* insertions Gus73 and Gus86. The activation of *hrp* genes was not affected by the pr-LPS treatment. The genes highly expressed during 5h of incubation in protected tissue were the same as those expressed in the unprotected tissue. Moreover the expression of *hrp* genes *in planta* was similar to expression in inducing medium.

Since pr-LPS treatment did not affect the expression of *hrp* genes either *in planta* or *in vitro*, we hypothesized that pr-LPS treatment could be having an indirect effect on *hrp* genes expression that was dependent on interaction between the bacterium and the plant. The protected tissue might react differently to a signal - codified by the *hrp* genes - sent by the bacteria to the plant. This interaction was studied by introducing into the wild-type strain of *E. amylovora* a plasmid containing the same Gus fusions, that previously were located in the chromosome. Two plasmids, p1055 and p1020, carrying the Gus73 and Gus86 insertions respectively, were eletroporated in Ea321 producing the two *hrp*⁺ mutants Ea321(p1055) and Ea(p1020). The GUS activity of these mutants was assayed *in vitro* and *in planta*. *In vitro* the Gus activity of both *hrp*⁺ mutants was not influenced by the presence of pr-LPS. In the protected tissue a significant decrease in Gus activity was found.

From this study, we draw four major conclusions. First, the pre-treatment of tobacco leaves with pr-LPS complexes protected against subsequent elicitation of the HR by *E. amylovora* without influencing the bacterial growth. Second, our results showed that pr-LPS complexes did not *per se* cause a nutritional effect that could have inhibited the *hrp* genes expression. Third, the activation of the *hrp* genes in the mutants with chromosomal Gus insertions was not influenced by the presence of pr-LPS either *in planta* or *in vitro*. Fourth, the *hrp* genes expression in the mutants with Gus insertions in the plasmid was significantly lower in the protected tissue. Therefore, our overall conclusion is that induced protection requires a completely functional *hrp* gene cluster so that the bacteria can send a signal to the plant, which following pr-LPS treatment react differently to this signal inhibiting the *hrp* genes expression.

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Loss of virulence of *Agrobacterium tumefaciens* following tumorigenesis on apple plantlets grown *in vitro*

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Abstract

The capacity to utilize opines produced by crown-gall tumors is conferred on virulent strains of *Agrobacterium tumefaciens* by the Ti plasmids. Various strains of *Agrobacterium* are avirulent, but nevertherless exhibit the capacity to utilize opines. In at least some of the avirulent agrobacteria, the opine catabolic potential is also encoded on plasmids. Because of these similarities, it seemed a possibility that virulent and avirulent strains of *A. tumefaciens* were related. Production of an endonuclease active on specific sequences of the Ti plasmid is induced at the onset of tumor induction. This further suggests that avirulent mutants are generated from the inciting, virulent agrobacteria as a result of tumorigenesis. To test this possibility, six different *A. tumefaciens* isolates recovered from apple, cherry or blackberry, as well as the well-characterized strain C58, were inoculated on cherry, pear, blackberry and apple plantlets grown *in vitro*. Bacteria were recovered from tumors which had developed one month after inoculation. One hundred bacterial clones recovered from each tumor were tested for virulence on tomato plants. Generally, all of the clones recovered from the various strain-plantlet combinations had retained virulence. However, up to 90% of the clones recovered from tumors on apple induced by the biotype 2, nopaline-type strain D10B/87 were avirulent. These avirulent clones still utilized nopaline and harbored a Ti plasmid. The Ti plasmid from the wild-type and mutant strains was transferred to a recipient *Agrobacterium*, which showed that, for 14 out of 15 mutants analysed, the mutation to avirulence resided on the Ti plasmid.

Introduction

Crown gall disease, to which most dicotyledonous plants are susceptible (DeCleen and DeLey, 1976), is induced by *Agrobacterium tumefaciens* . This bacterium harbors a large plasmid called pTi which is responsible for virulence (Van Larebeke et al., 1974) One of pTi regions, called T-DNA, is transferred and integrated into the plant genome where it produces proteins involved in production of phytohormones responsible for tumor development. A second part of the T-DNA is responsible of the production of new compounds called opines. In addition, genes present in a nontransfered region of the Ti plasmid confer on the bacterium the capacity for catabolism of opines produced by transfomed cells (Reviewed by; Kado, 1991; Ream, 1989). In nature, many *Agrobacterium* strains unable to produce disease (*A. radiobacter*) nevertheless exhibit capacity to catabolize opines. In some cases, this ability is conferred by plasmids showing some homology with pTi (Merlo and Nester, 1977). This suggest that avirulent opine utilizing strains could appear following mutations in virulent agrobacteria. These mutation would inactivate virulence without interfering with opine catabolism functions.

The virulence (*vir*) region of pTi is induced by phenolic compounds produced at plant wounds (Stachel et al., 1985; Spencer and Towers, 1988). It is then responsible for the production of proteins which promote excision of the T-DNA from the Ti plasmid, and transfer of this DNA from the bacterium to the plant cell. One of the *vir* genes (virD) codes for a site-specific endonuclease acting on border sequences flanking the T-DNA (Yanofsky et al., 1986). We have hypothesized that under induction conditions, avirulent mutants could be produced as a result of endonuclease or other type of genetic activity promoting rearrangements of the Ti plasmid. The reality of a such phenomenon has been demonstrated recently by studying the effect of *vir* phenolic inducers on the nopaline-type strain *A. tumefaciens* C58 (C. Fortin, unpublished).

In the present work, we have studied the occurrence of Ti plasmid rearrangements following tumorigenesis on various types of plantlets.

Materials and Methods

Bacterial strains. The *Agrobacterium tumefaciens* strains which were used in this stydy are listed in Table 1. For greenhouse studies, a spontaneous rifampicin resistant mutant of D10B/87 was used.

Plant inoculations. Sterile plantlets of cherry, pear, blackberry and apple were cultivated on tissue culture media modified from Murashige and Skoog

Table 1. *Agrobacterium tumefaciens* strains used in this study.

Strain	Host of origin	Biotype	Opine Utilization
C58	Cherry	I	Nopaline
B49C/83	Apple	II	Nopaline, Mannopine
D10B/87	Apple	II	Nopaline
I11/85	Cherry	II	Nopaline
I22/85	Cherry	II	Nopaline
B209B/85	Blackberry	ND(a)	ND
B230/85	Blackberry	ND	ND

(a) ND, not determined

according to the species of plant. *In vitro*-grown plants were maintained at 25°C with a 16h photoperiod. Greenhouse-cultivated apple planlets came from *in vitro* culture and were placed under greenhouse conditions two weeks before inoculation. Plantlets were inoculated by piercing their stem with a needle previously dipped in a bacterial colony. After inoculation, *in vitro* and greenhouse grown plantlets were returned to their respective incubation conditions.

Recovery of bacteria from crown gall tumors. Following tumor development (one to three months), the tumor tissue was macerated in 2 ml of saline solution for 30 min. Dilutions were plated on rich medium. For greenhouse studies rifampicin (50 µg/ml) and cycloheximide (20 µg/ml) were added to the medium used for bacterial recovery. From each tumor, one hundred bacterial colonies were selected for further testing.

Determination of virulence and nopaline utilization. Bacterial virulence was tested in the greenhouse on tomato plants (Bonnie Best or Vendor) by stem inoculation three to four weeks after sowing. The capacity for nopaline utilization was examined in liquid AT medium containing nopaline (800 mg/L). After 72h of incubation at 28°C under shaking (175 rpm), development of turbidity indicated a positive result.

Ti plasmid transfer. The Ti plasmid was transfered from a donnor *Agrobacterium* strain to the recipient strain C58C1rs. This recipient had been cured of the Ti plasmid and was resistant to streptomycin and rifampicin. Selection was made on AT medium containing nopaline (800 mg/L), rifampicin and

streptomycin. The transconjugants were tested for virulence as described above.

Results

In vitro-grown plants of cherry, pear, apple and blackberry were inoculated with the various bacteria listed in Table 1 and then maintained under conditions of *in vitro* culture. After one month, tumors were excised and bacteria were recovered. One hundred clones of each parental strain were tested for virulence.

Out of the seven srains tested in this manner, D10B/87 produced avirulent clones. However, these avirulent mutants were obtained only on Mark apple rootstocks. No D10B/87 mutants were recovered from cherry, pear or blackberry. On two independent tests, avirulent mutants represented respectively 90% and 35% of the clones tested. All of the clones were able to utilize nopaline as the sole source of carbon and nitrogen (Table 2).

Following these initial results, other types of apple rootstocks were also inoculated with D10B/87. Three months after inoculation, tumors had become visible on the various rootstocks and bacterial clones were recovered from each of these. Clones were recovered either from the internal tussues of the tumor (with the gall surface removed and following maceration as described above) or else from the tumor surface. In this case, bacteria were recovered simply by washing the surface of the gall with saline solution and plating this solution as

Table 2. Production of avirulent mutants following tumorigenesis.

Apple rootstock inoculated	Rootstock growth conditions	Proportion (%) of avirulent clones recovered from tumor:		Proportion (%) of nopaline utilizing clone recovered from tumor:	
		surface	inner tissue	surface	inner tissue
Mark	In vitro	ND (a)	90	ND	100
Ottawa3	In vitro	99	98	100	100
P106	In vitro	68	95	100	99
BUD116	In vitro	82	90	98	100
Ottawa3	Greenhouse	0	0	100	100
Malling7	Greenhouse	0	0	100	100

(a) ND, not determined

described above. Table 2 shows that tumors formed by the three apple rootstocks contained up to 99% of avirulent mutants. Most of the clones recovered from the tumors had retained capacity for nopaline catabolism. Similar results were obtained from clones recovered from the tumor inner tissue or from the tumor suface. These results confirmed previous observation on the presence of avirulent mutants in apple tumors (see above) and showed that mutant production was not a direct consequence of tissue maceration diring bacterial recovery. Inoculation of strain D10B/87 in tissue culture medium in the absence of plantlets did not induce mutagenesis.

In vitro culture may alter various aspects of plant metabolism (Ibrahim, 1987; Hegedus and Phan, 1983), including metabolism of phenolic compounds and photosynthesis. In addition, chemical composition of the plant is likely to be affected. Thus the mutagenic potential of *in vitro* cultivated and greenhouse grown plants was compared. Strain D10B/87 was inoculated on two different apple cultivars grown in the greenhouse. After three months, bacteria were harvested from tumors and tested for virulence. No mutants were obtain under these conditions (Table 2).

Fifteen avirulent mutants obtained from tumors on *in vitro*-grown Mark apple rootstocks were further analysed and compared to the wild type D10B/87. Eckhardt gel analysis showed that strain D10B/87 harbors two plasmids. The larger of these was transferred to the recipient *Agrobacterium* C58C1rs to establish that this particular plasmid encoded nopaline catabolism and virulence function. This pTiD10B was of about the size of pTiC58. No deletions in the pTi were detected in the fifteen mutants analyzed (data not shown).

Table 3. Localization of the mutation to avirulence of D10B/87 clones recovered from apple tumors produced *in vitro*.

Clone of D10B/87	Virulence of clone	Virulence of transconjugant	Mutation on Ti plasmid
Wild type strain	+	+	NA (a)
14/15 avirulent mutants	-	-	Yes
1/15 avirulent mutant	-	+	No

(a) NA, not applicable.

The plasmidic or chromosomal localization of the mutations to avirulence was also examined by conjugative transfer of the Ti plasmid from the D10B/87 mutants to the avirulent C58C1rs recipient. The resulting transconjugants were tested for virulence on tomato plants. Transfer of the Ti plasmid from the wild type D10B/87 to C58C1rs produced a virulent transconjugant (Table 3). However, transfer of the Ti plasmid from fourteen out of the fifteen avirulent mutants analyzed failed to confer virulence on the C58C1rs recipient. This indicated that, in the case of these fourteen mutants, the mutation to avirulence resided on the Ti plasmid.

Conclusions

In this work, avirulent mutants were obtained following tumorigenesis under conditions of *in vitro* culture. Out of seven strains tested, only one of these, strain D10B/87, produced mutants on various types of apple rootstocks. This strain did not yield mutants on three other plant species. Most of the avirulent mutants retained the capacity to catabolize nopaline. Mutagenesis was limited to conditions of *in vitro* culture. This may correspond to altered plant physiology and composition induced by *in vitro* culture. Out of fifteen mutants analyzed, fourteen were mutated on pTiD10B.

Results from this stydy suggest that, under particular conditions, tumor induction by some strains of *A. tumefaciens* can be accompanied by the massive production of avirulent mutants.

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Fingerprint analysis of plasmids of *Pseudomonas solanacearum*

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ABSTRACT

Sixteen isolates of *Pseudomonas solanacearum* were screened for plasmids. They harboured 1-3 plasmids but most of the isolates contained a single plasmid. Fingerprinting and cluster analysis of plasmids revealed that isolates of *P. solanacearum* could be grouped, based on race.

KEYWORDS

Plasmid, Fingerprint, Cluster analysis.

INTRODUCTION

Several methods of DNA analysis have been used to compare and identify plant-pathogenic bacteria. These include DNA-DNA hybridisation, restriction fragment length polymorphism (RFLP), plasmid profile analysis, genomic fingerprinting and plasmid fingerprinting (COOKSEY & GRAHAM, 1989). Each method has its own limitation depending on the specificity of comparison desired and the chemical complexity of the procedure required to analyse.

Plasmids with very different digest pattern may have extensive homology when analysed by DNA-DNA hybridisation (QUANT & MILLS, 1981) suggesting that digest patterns are a more sensitive measure of variation in base sequences. Different restriction endonuclease recognition sites occur at different frequencies in *Pseudomonas* plasmids. The EcoR1 has more sites than BamH1 and HindIII (SZABO *et al.*, 1981 ; QUANT & MILLS, 1984 ; MORALES & SEQUEIRA, 1985).

In this paper we have surveyed isolates of *Pseudomonas solanacearum* from different geographical regions for plasmid profile. Fingerprinting of plasmids from 10 isolates of *P. solanacearum* was done.

MATERIALS AND METHODS

Cultures

Bacteria were isolated from infected plant materials collected from different regions of India and identified according to BRADBURY (1986).

Plasmid isolation

Cultures identified as *Pseudomonas solanacearum* were screened for plasmid profile by employing the alkaline lysis method (BOOMINATHAN & MAHADEVAN, 1988).

Restriction enzyme digestion of plasmid DNA

EcoRI was obtained from Promega, Leiden, The Netherlands. Five µg of plasmid DNA from 10 isolates were digested individually with Eco RI. Number of fragments and size of the fragments were individually compared and cluster analysis was done with the standard method (GOWER, 1985). Similarity matrices were formed for all pairwise combinations of strains using simple matching. Unweighted pair group mean averages (UPGMA) and average linkage hierarchical clustering methods were used.

RESULTS

Plasmid profile in *Pseudomonas solanacearum*

Alkaline lysis method facilitated the detection of both large and small plasmids in all the strains (Table 1). Out of 16 isolates of *P. solanacearum*, 11 contained a single plasmid, 4 isolates (PS2, PS6, PS18, PSG2) harboured 2 plasmids and in PS5, 3 plasmids were detected. *P. solanacearum* displayed diversity in the distribution of plasmids.

Fingerprinting of *P. solanacearum* plasmids

To study the similarity of different isolates of *P. solanacearum*, plasmid fingerprint analysis was carried out. The reproducibility of fragmenting pattern for the strains indicated that this approach is useful to analyse intrapathovar variation.

Table 1. Plasmids in *Pseudomonas solanacearum*

Spacies	Race	Isolate No.	Number of plasmids
P. solanacearum	3	PS1	1
P. solanacearum	3	PS2	2
P. solanacearum	3	PS3	1
P. solanacearum	3	PS4	1
P. solanacearum	3	PS5	3
P. solanacearum	3	PS6	2
P. solanacearum	3	PS7	1
P. solanacearum	3	PS18	2
P. solanacearum	3	PSCPRI	1
P. solanacearum	1	PSBR1	1
P. solanacearum	1	PSBR2	1
P. solanacearum	1	PSBR3	1
P. solanacearum	1	PSG1	1
P. solanacearum	1	PSG2	2
P. solanacearum	1	PSBI	1
P. solanacearum	1	PSCHI	1

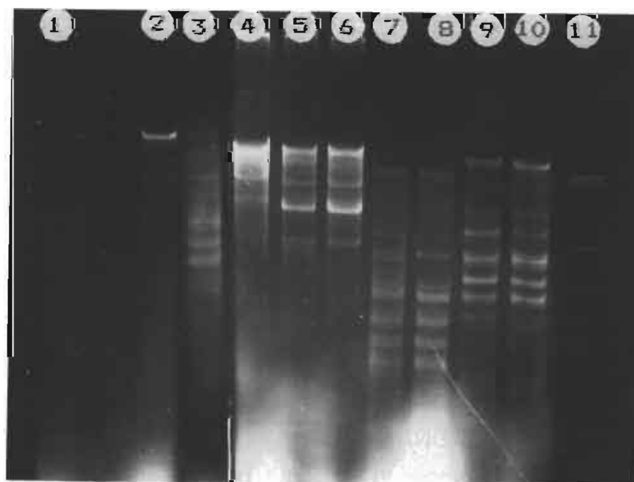

Fig.1. EcoRI fragment pattern of plasmids from *P.solanacearum* 1.PS18, 2.PSBI, 3.PSCPRI, 4.PSBR1, 5.PSBR2, 6.PSBR3, 7.PS1, 8.PS2, 9.PS3, 10.PS4, 11.λ Hind III.

Ten isolates were randomly selected and their plasmids were digested with EcoRI. Fragment patterns are presented in Fig 1. *P. solanacearum* plasmids had distinct fragment pattern.Race 3 isolates had fairly similar fingerprints, isolates PS1, PS2, PS3, PS4, PS18 and PSCPRI produced 11, 11, 9, 14, 10 and 10 fragments, respectively. Fragments ranged from 3 Kb to 98 Kb in size. All the race 3 isolates shared common fragments of 15, 12, 10 and 7 Kb. Isolates PS18 and PSCPRI shared indigenous fragments of 23 and 20 Kb, the remaining 4 isolates had common fragments of 41 and 29 Kb. The 4 isolates PSBR1, PSBR2, PSBR3 and PSBI produced 4, 5, 8 and 4 fragments respectively and their sizes ranged from 5 to 100 Kb. The race 1 isolates PSBR1, PSBR2 and PSBR3 shared common fragments of 51,41 and 23 Kb.

Cluster analysis

Cluster analysis carried out by using UPGMA indicated that isolates of *P. solanacearum* displayed variation. Based on similarity coefficients, similarity matrix was constructed. *P. solanacearum* had 9 mutually low pairs each of which was picked up from the similarity coefficient table based on similarity.

Cluster analysis of similarity, based on restriction enzyme fragments indicated the presence of two distinct groups (Fig. 2). The first group consisted of PS1, PS2, PS3, PS4, PS18 and PSCPRI, only the isolates of race 3.

The second group contained PSBR1, PSBR2, PSBR3 and PSBI including the isolates of race 1.

DISCUSSION

The isolates contained 1 to 3 plasmids. MORALES & SEQUEIRA (1985) reported that out of 39 strains of *P. solanacearum*, 22 contained 1-2 plasmids. According to BOUCHER *et al.* (1986), virulence genes were located on a mega plasmid in *P. solanacearum*. A mega plasmid isolated from *P. solanacearum* was implicated in the production of catechin 1,2 dioxygenase, that cleaved catechin (BOOMINATHAN *et al.*, 1989).

The high specificity of DNA fingerprinting is a limitation since only closely related bacteria can be compared. We did not attempt to compare strains of different pathovars, but we did compare the isolates of *P. solanacearum*.

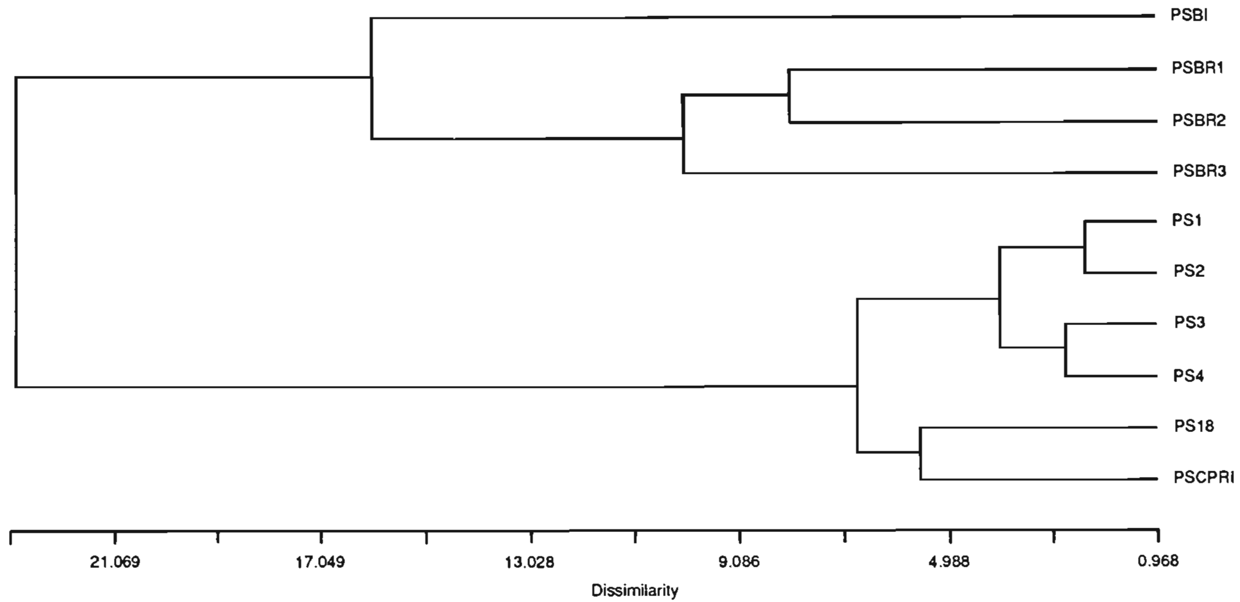

Fig.2. Dendrogram obtained by cluster analysis of similarity coefficients from EcoRI plasmid finger prints of 10 isolates of *P. solanacearum*.

DNA fingerprinting and cluster analysis of isolates of *P. solanacearum* plasmids provided that the race 3 isolates grouped into one and the race 1 isolates formed a separate group despite geographical distribution. The isolates of race 3 displayed high degree of similarity in their banding pattern. Isolates of race 1 showed high variation in their fragment pattern but the isolates clustered in one. COOKSEY & GRAHAM (1989) found that genomic fingerprints of *P. syringae* pv. *tomato* were placed under two groups, one resistant to copper and the other sensitive to copper regardless of geographical distribution. Cluster analysis of restriction banding pattern of plasmids from *P. syringae* pathovars grouped all strains of pathovar *pisi* separately from pv. *glycinea*, pv. *phaseolicola* and pv. *syringae* (KING, 1990).

ACKNOWLEDGEMENTS

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Inhibition of compatible/incompatible reaction between *Xanthomonas campestris* pv. *malvacearum* and cotton

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INTRODUCTION

The induction period of the incompatible hypersensitive reaction (HR) is much shorter (20-48 hrs) than that of the compatible/susceptible reaction (SR in 3-10 days). The HR is induced only at high initial inoculum concentration (10^6 cfu/ml) but does not result in high population of the bacteria in the intercellular spaces. The SR can be induced at low population ($10^2/cm^2$ or more) indicating prolonged multiplication in host tissue (KLEMENT, 1972 ; ERCOLANI, 1984 ; VERMA, 1986). The main purpose of this investigation was to study the interaction between cotton (*Gossypium hirsutum*) and (*X. c.* pv. *malvacearum*) *Xcm* and to determine the critical periods required for SR/HR, chemical inhibition of SR/HR, various types of HR/SR, interaction of genotypes (races) of *Xcm* and how these studies could be exploited in the integrated management of bacterial blight of cotton.

MATERIAL AND METHODS

A 24 hr old culture of *Xcm*R-32 (Race 32) grown on nutrient sucrose agar medium and possessing the virulence factors for five bacterial blight resistant genes namely B-7, B-4, B-2, B-In & B-N, was used for inoculations by syringe infiltration (VERMA, 1986) at 10^7 cfu/ml. The main cotton cvs/differentials used were Acala-44 (with no gene for resistance and showing SR) and 101.102-B (possessing B-2 + B-3 + unknown and showing HR to all the races described from India).

RESULTS AND DISCUSSION

Cotton genotypes and their reaction to different races of *Xcm*

The HR could be distinguished into at least five grades of reactions namely, no reaction, light greenish lesion, light yellow lesion, light brown yellowish and brown lesion completely dry and papery. The SR could be grouped into the following tree types : central area of WS turned brown, central area turned necrotic and the entire area turned brown and dry. SR occurred in 3-6 days and resulted in high *Xcm* population in the host, while HR required only about 20 hr and the multiplication of *Xcm* was restricted in 101.102.B. On other cvs the HR occured in 40-72 hrs. For example, *Xcm*R-8 (race 8) produced HR within 40-44 hrs on 1-10B (containing gene B-In) and within 48 hrs on Stoneville-20 (containing gene B-7) ; whereas *Xcm*R-1-V$^-$ (avirulent race 1) produced HR on 1-10.B in 72 hrs.

It is apparent that the same race was capable of possessing both HR and SR factors, the expression of which depended on the genotype of the host. It was, however, interesting that *Xcm*R-32 which produced SR on all cvs except 101.102.B (where HR was produced) yielded a streptomycin resistant avirulent isolate (*Xcm*R-32-str^{r}V$^-$) which did not induce any reaction on susceptible cvs, but retained HR on 101.102.B, indicating that in this mutant the antibiotic affected the genes responsible for SR but not the genes responsible for HR. Thus, the genes responsible for SR and HR are not only different but located at such distance where the loss of SR-genes (pathogenic factor) does not have any effect on HR-genes (the virulence factor). However, another avirulent mutant (not streptomycin resistant) *Xcm*R-1-V$^-$ lost its SR on Acala-44 but did produce HR on Acala-44 ; the mutant also retained its HR capability on other cvs. The reason for this is not clear because HR was produced on a SR reacting cv ; but this may have to do something with the fact that Acala possesses no gene for resistance to bacterial blight.

If HR (incompatibility) is accepted (ELLINGBOE, 1982 ; VERMA, 1992) as the specific recognition event (between host-pathogen) then the less virulent race (which produces HR on most of the cvs, e.g. *Xcm*R-1) must be regarded as possessing the recognition factors to a larger number of cvs than the highly virulent race of *Xcm*. However, the SR also must depend on specificity because the symptoms are produced only on restricted number of cvs (for example *Xcm* produces symptoms on certain cvs of cultivated species of cotton but not on so many other plants which are non-hosts). It appears that the recognition phenomenon is functional at various levels. Level-0 shows no recognition and symptoms are not produced (e.g. non hosts). At level-1 there is some recognition (e.g. the hosts) and symptoms are produced. At level-2 extreme (specific) recognition is there and too much symptoms are produced in relatively short time so that browning and tissue collapse are rapid enough to inhibit the further spread

of the pathogen. Thus, level 1 recognition would lead to SR while level 2 recognition would lead to HR. These different levels of recognition could be under the specific control of different genes. There is also a possibility that the same group/cluster of genes may behave differently under variable regulatory control (VERMA, 1992).

It is generally accepted that phytopathogenic bacteria interact with their hosts in two main ways i.e. saprophytic (resident/epiphytic phase) and pathogenic (SR/HR) ways involving a large number of fitness genes capable of periodic adaptations (VERMA, 1986). It is possible that the initial population contains a mixture of better saprophytes and better pathogens. During pathogenesis (i.e. spread of disease during season) the pathogens multiply faster and the saprophytes are reduced in number. Thus, there is a selection for "virulence" within the genetic potential of the total population. It is interesting that the observed frequency (OF) of *Xcm*R-32 was 42 whereas the expected frequency (EF) was 0.16 (DUTTAMAJUMDER & VERMA, 1992). In case of *Xcm*R-31 the figures were 0.0025 (OF) and 0.012 (EF), clearly indicating that the selection pressure was favouring race-32 genotype, but not the genotype of race-31 (race -31 is virulent for the same oligogenes as race-32, i.e. B-7, B-2, B-In & B-N ; but race-31 is not virulent for polygenes i.e. B-Sm of Stoneville-2B-S9). Thus the genotypes of *Xcm* virulent for polygenes become more fit in the environment (VERMA, 1986). Further, the acquisition of virulence for oligogenes makes the pathogen most fit simultaneously.

Interaction amongst genotypes of *Xcm* and cotton

Several genotypes (races) of *Xcm* were present in single lesion, particularly on leaf lesions in Acala-44, a differential cv. with no genes for resistance to bacterial blight, and where most of the races could multiply individually. The reaction of mixed races (genotypes) of *Xcm* was synergistic on Acala-44, the increase in lesion area ranged from 48-73 % at 14 days after the inoculation. The reaction of the virulent genotype was not changed in a mixture of races at 1:1 ratio (for example *Xcm*R-32 behaved as *Xcm*R-32 in a mixture of *Xcm*R-32 and *Xcm*R-8 ; *Xcm*R-8 is less virulent and virulent to only B-In-gene). However at 2:1 ratio of less virulent : virulent genotype (i.e. *Xcm*R-8 : *Xcm*R-32) on cvs which are resistant (show HR) to the less virulent race, the mixture also reacted as HR, indicating that HR dominated, started earlier and inhibited the SR. In the presence of avirulent isolates also the virulent isolate could not exhibit its properties, i.e. the mixture behaved as avirulent and induced a HR. The results emphasised that the HR may be used by the host to eliminate or curtail the development of certain races in mixed infections. It was generally concluded that the disease reaction against mixed races of *Xcm* was synergistic or mixed on cvs susceptible to both the races, but the reaction was hypersensitive/antagonistic if one of

the races of the mixture was incompatible to the cv under test. The results also point out the dangers of the use of race-mixtures for screening of segregating populations in resistance breeding programmes.

Chemical inhibition of HR/SR

Challenge inoculation of chemicals into areas pre-inoculated (pre-infiltrated by syringe) with *Xcm* inhibited SR on Acala-44 as well as HR on 101.102.B up to a certain period. Of the chemicals used, four (bacitracin, chlorotetracycline, cycloserine and streptomycin) inhibited HR only upto 7 hrs challenge but not at 9 hrs. It was concluded that this 7hrs period was critical and live/viable *Xcm* were needed only for 7 hrs after which the reaction was committed to completion (of HR). Cysteine did not inhibit HR or SR. Cycloserine was not effective against SR, but the other bacterial inhibitors (bacitracin and streptomycin) inhibited SR completely except chlorotetracycline which was not effective at 9 hrs challenge.

HR in soybean leaves to incompatible pseudomonads could be inhibited by challenging streptomycin and blasticidin upto 9 hrs (KEEN *et al.*, 1981). HR in pepper leaves by *X.c.* pv. *vesicatoria* was prevented by challenge of chloramphenicol , rifampicin and tetracycline up to 3 hrs on pre-inoculated *X.c.* pv. *vesicatoria,* but challenge after 5 hrs did not inhibit HR (MEADOW & STALL, 1981). FOLLIN (1983) demonstrated that when *Xcm* was suspended in cycloheximide (5 and 10 µg/ml), there was no effect on SR or HR on cotyledons of Acala-44 or 101.102.B. However, when *Xcm* was suspended in actinomycin-D (10-20µg/ml) in an aqueous solution of mannitol of 400 µg/ml, which inhibits DNA-dependent RNA polymerase, the SR was inhibited (that is, Acala-44 reacted as 101.102.B, i.e. HR), while there was no effect on HR.

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Investigations on the molecular weight of alginate synthesized by *Pseudomonas syringae* pv. *phaseolicola* races 1 and 2

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ABSTRACT

Alginate, produced by two strains of *Pseudomonas syringae* pv. *phaseolicola* (races 1 and 2) *in vitro*, was purified by ion exchange chromatography. Fractions of the alginate peak were investigated by polyacrylamide gelelectrophoresis and gelpermeation chromatography. Determinations of the molecular weights of alginate from the two strains did not reveal big differences.

INTRODUCTION

Most phytopathogenic bacteria produce slimes such as extracellular polysaccharides (EPS) or glycoproteins when grown *in planta* or *in vitro*. *Pseudomonas syringae* pv. *phaseolicola*, the incitant of "halo blight" of bean (*Phaseolus vulgaris*) sythesizes two kinds of EPS, levan and alginate (GROSS and RUDOLPH, 1987 a,b). Since the degree of water-soaking in bean leaves during the compatible interaction was correlated with the alginate production *in vitro* (NEUGEBAUER et al., 1990), alginate may be involved in pathogenesis.

However, the function of alginate *in planta* has not been completely elucidated. Alginate may prevent a close cell-to-cell contact of host and parasite, which is necessary for the initiation of plant resistant reactions (STALL and COOK, 1979).

Differences in virulence of the two races of *Pseudomonas syringae* pv. *phaseolicola* might be partly explained by the quality and quantity of the synthesized alginate. Previous analysis of alginate showed homogeneity at the chemical level (NEUGEBAUER et al., 1991), but the molecular weight was not determined.

MATERIALS AND METHODS

Two strains of *Pseudomonas syringae* pv. *phaseolicola* obtained from the Göttinger Sammlung Phytopathogener Bakterien (GSPB), GSPB 567 (race 1) and GSPB 1715 (race 2), were cultured at 25 °C *in vitro* in a liquid suspension with sucrose as carbon source.

After two days EDTA (10 mM final concentration) was added and the bacteria were removed by centrifugation (10000 xg, 15 min), the supernatant was filtered (membrane filter 0.45 μm) and directly fractioned by ion exchange chromatography (IEC) at pH 6.0 using TMAE-Fractogel (Merck). Alginate and levan were determined according to BITTNER and MUIR (1962) and HELBERT and BROWN (1955), respectively.

Polyacrylamide gelelectrophoresis (PAGE) was performed with fractions of the alginate peak after IEC. Gels were stained with 1 % toluidine blue for two minutes and destained with water.

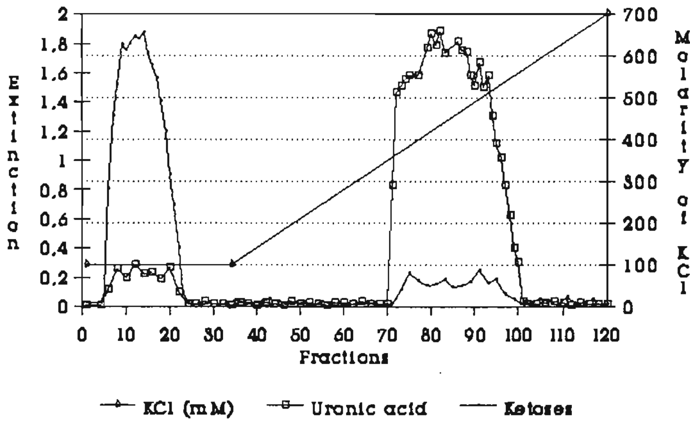
Several fractions of the alginate peak of IEC were pooled and compared by gelpermeation chromatography (GPC) using Fractogel TSK HW 65 (Merck).

RESULTS AND DISCUSSION

Separation of the two exopolysaccharides by IEC was an excellent way to quickly obtain purified alginate from the supernatant of bacterial supensions (Fig. 1). Moreover, yield of alginate obtained by this method was much higher than in previous investigations (GROSS and RUDOLPH, 1987a) so that we were able to obtain approximately 1 g purified alginate from 1 l culture suspension.

PAGE showed that the early eluting fractions of the alginate peak seemed to be of lower molecular size than the later eluting ones (Figs. 2 and 3). However, no big differences appeared to exist in the molecular weight of alginate from these two strains. In tendency the alginate from GSPB 567 (race 1) might contain more polymers of lower molecular weight than that from GSPB 1715 (race 2).

These results were confirmed by GPC. The early eluting fractions of IEC contained low molecular alginate whereas later eluting fractions contained high molecular alginate. Comparison of the two strains led to the impression that



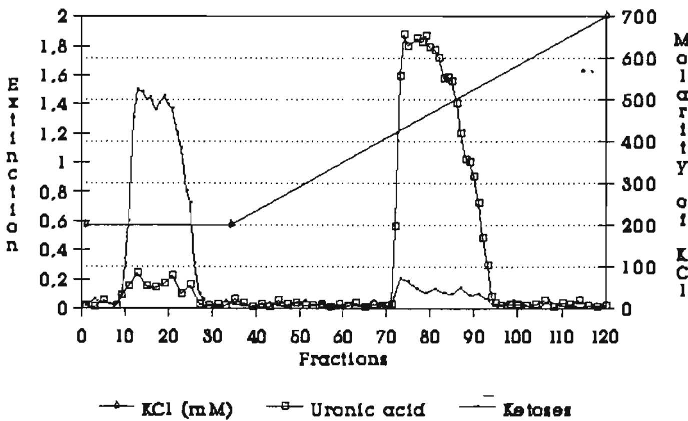
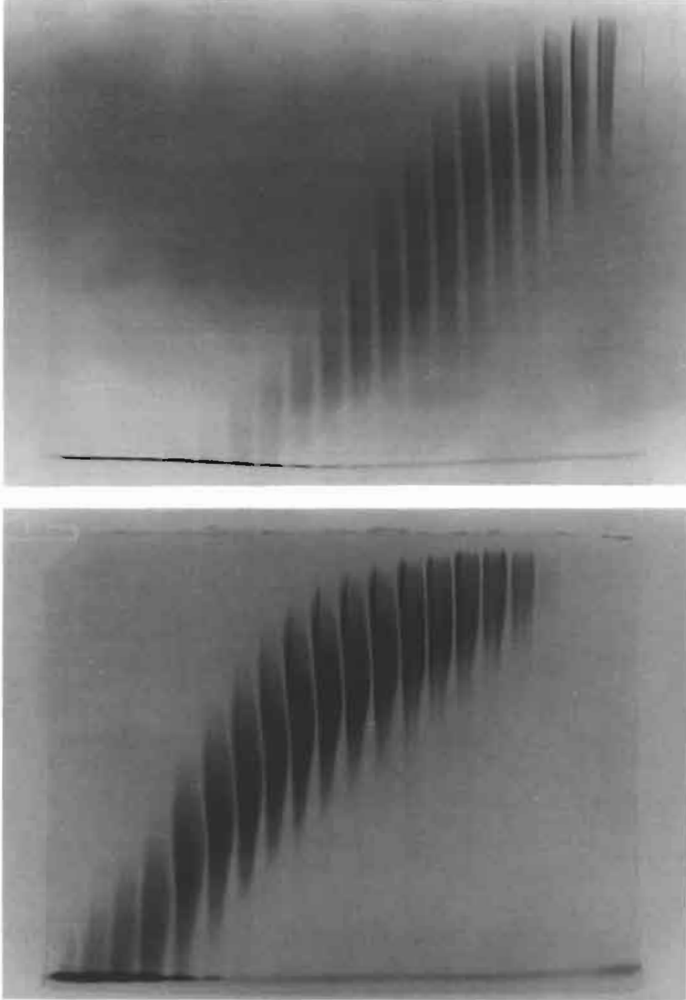


Fig. 1: Purification of alginate by ion exchange chromatography

alginate of GSPB 567 (race 1) contained a higher amount of low molecular weight polymers. Nevertheless, the main alginate of the two strains eluted closely together (Fig. 4).

The studies, thus, showed that strains of races 1 and 2 of *P.s.* pv. *phaseolicola* have the ability to synthesize highly polymerized alginate *in vitro*. Further investigations are planned to analyse the synthesis of alginate and its degree of polymerisation during pathogenesis *in planta*.



Figs.2 and 3: Polyacrylamide gelelectrophoresis of the alginate peak after ion exchange chromatography from *P.s.* pv. *phaseolicola* strains GSPB 567, race 1 (above), and GSPB 1715, race 2

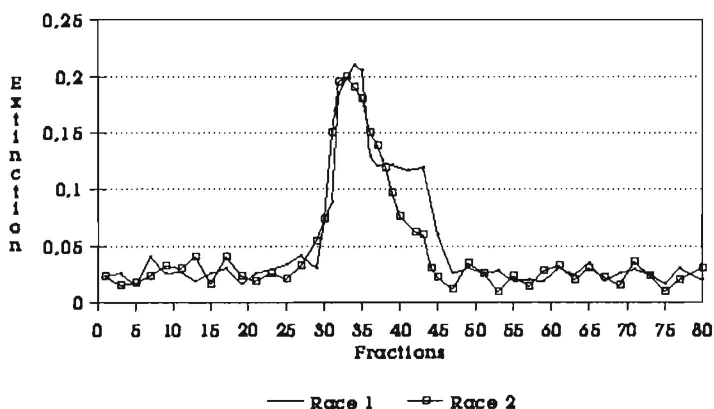


Fig.4: Comparison of alginate produced by GSPB 567 and GSPB 1715 investigated by gelpermeation chromatography

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Aspects of alginate biosynthesis by *Pseudomonas syringae* pv. *phaseolicola* First investigations

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Investigations on alginate biosynthesis by the human pathogenic bacterium *Pseudomonas aeruginosa* and marine brown algae have shown that alginate is synthesized via the sequence fructose-6-P → mannose-6-P → mannose-1-P → GDP-mannose → GDP-mannuronic acid → → → extracellular alginate. The enzymes phosphomannose isomerase, phosphomannomutase, GDP-mannose pyrophosphorylase, GDP-mannose dehydrogenase and further enzymes for polymerization, acetylation and epimerization catalyse these reactions. For our investigations we used crude protein extracts from *Pseudomonas syringae* pv. *phaseolicola* strain 1321 grown with mannose. After a long incubation time we found a specific activity of phosphomannose isomerase of 0,06 mU/mg. For the reaction mannose-1-P → mannose-6-P we determined after a long incubation time a specific activity of 0,47 mU/mg. The catalysis of this reaction seems not to be due to a "phosphomannomutase" but to the constitutive phosphoglucomutase. The formation of GDP-mannuronic acid from GDP-mannose by a NAD-dependent GDP-mannose dehydrogenase seems to occur.

Keywords: *Pseudomonas syringae* pv. *phaseolicola*, alginate biosynthesis, phosphomannose isomerase, phosphomannomutase, phosphoglucomutase, GDP-mannose dehydrogenase

Pseudomonas syringae pv. *phaseolicola* (*P. s.* pv. *ph.*) produces the extracellular polysaccharide alginate consisting of mannuronic acid and its C_5-epimer guluronic acid. The mannuronic acid can be acetylated. Alginate seems to be a

factor of pathogenicity and virulence for the formation of disease symptoms on bush bean (*Phaseolus vulgaris*). For instance alginate contributes to formation of water soaking and protects the bacterium like a "mask" from the recognition by plant cells (GROSS, 1987). Investigations on alginate biosynthesis by the human pathogenic bacterium *Pseudomonas aeruginosa* showed that alginate is synthesized by the sequence fructose-6-P→ mannose-6-P→mannose-1-P→ GDP-mannose → GDP-mannuronic acid → → → extracellular alginate. The enzymes phosphomannose isomerase (PMI), phosphomannomutase (PMM), GDP-mannose pyrophosphorylase (GMP), GDP-mannose dehydrogenase (GMD) and further enzymes for polymerization, acetylation and epimerization catalyse these reactions (SA-CORREIA *et al.*,1987; MAY *et al.*, 1991). Similar results were found by investigations on alginate biosynthesis in marine brown algae (LIN & MASSID, 1966). Based on these results we followed the working hypothesis that *P. s.* pv. *ph.* also synthesizes alginate according to the described sequence. Nevertheless another biosynthetic sequence is also conceivable: the direct oxidation of mannose to mannuronic acid and its following activation. Up to now the following reaction steps have been checked for *P. s.* pv. *ph.*:

1) conversion of fructose-6-P into mannose-6-P
2) conversion of mannose-6-P into mannose-1-P
3) NAD-dependent oxidation of GDP-mannose in connection with formation of GDP-mannuronic acid
4) direct oxidation of mannose in connection with formation of mannuronic acid

MATERIAL AND METHODS

Bacterial strain and preparation of crude protein extract.

*P. s.*pv. *ph.* NCPPB 1321 was grown in a synthetic culture broth containing per litre: 5.5 g Na_2HPO_4, 2.6 g KH_2PO_4, 2 g NH_4Cl, 1 g $NaSO_4$, 0.1 g $MgSO_4$, 0.01 g $FeSO_4$, 0.01 g $MnSO_4$, 5 g mannose. Bacteria were harvested by centrifugation, disrupted by ultrasonics, centrifuged at 20000 x g for 30 minutes and the supernatants were dialysed against 0.01 M MOPS buffer, pH = 7.0 for three days. Protein content was measured by the method of Bradford (1976).

Assays

PMI. The incubation assay (incubation temperature of 22°C) contained 10 mM MOPS buffer, pH=7.0, 10 mM $MgCl_2$, 3 mM mannose-6-P, 300 µl (c. 5 mg protein) of crude protein extract in a total volume of 4.5 ml. The control assay contained denatured crude protein extract. The samples (1 ml) after 0, 4, 8 h were deproteinized by heating and centrifuged. In all samples fructose-6-P and mannose-6-P were determined enzymatically by the following assay: 10 mM MOPS buffer, pH=7.0, 1 mM NADP, 0.1 ml sample in a total volume of 1.5 ml. By gradual addition of 5-10 units each of glucose-6-P dehydrogenase (EC 1.1.1.49), phosphoglucose isomerase (EC 5.3.1.9) and phosphomannose isomerase (EC 5.3.1.8) and monitoring the rate of $NADP^+$ reduction at 334 nm at 24°C it was possible to determine the quantity of these substances.

PMM. The PMM-activity was measured by the following optical enzyme assay: 10 mM MOPS buffer, pH=7.0, 10 mM $MgCl_2$, 0.25 mM glucose-1.6-bis-P or 0.17 mM mannose-1.6-bis-P, 1 mM NADP, 0.1 ml (c. 5 mg protein) crude protein extract respectively 5 units phosphoglucomutase (EC 5.4.2.2), 5-10 units each of phosphomannose isomerase, phosphoglucose isomerase and glucose-6-P dehydrogenase. The reaction was started by the addition of 1 mM mannose-1-P (total volume of enzyme assay 1.5 ml). The rate of $NADP^+$ reduction was measured by recording increases in optical density at 334 nm at 24°C. The long time incubation assay (incubation temperature of 22°C) contained 10 mM MOPS buffer, pH=7.0, 10 mM $MgCl_2$, 1 mM mannose-1-P, 0.175 mM mannose-1.6-bis-P, 300 µl (c. 5 mg protein) of crude protein extract in a total volume of 4.5 ml. The control assay contained denatured crude protein extract. The samples (1 ml) after 0, 1, 2, and 4 h were deproteinized by heating and centrifuged. The half volume of each sample was hydrolized with 0.2 N H_2SO_4 at 100°C for 30 min. In the unhydrolized and hydrolized samples mannose-6-P, mannose-1.6-bis-P and glucose-1.6-bis-P were detected enzymatically as described under PMI. The assay for the detection of mannose-1-P additional contained 2.53 mM ATP and 10 units hexokinase.

GMD. The incubation assay for demonstration of GDP-mannose dehydrogenase activity (incubation temperature of 22°C) contained 10 mM MOPS buffer, pH=7.5, 2.5 mM NAD, 5 mM dithiothreitol, 10 mM $MgCl_2$, 2.5 mM GDP-mannose, GDP-[U-^{14}C]mannose (0.037 MBq), 1 ml (c. 22 mg protein) of crude protein extract in a total volume of 3 ml. The control assay contained denatured crude protein extract. The samples (1 ml) after 0 and 24 h were deprotei-

nized by treatment with Centrisart I ultrafiltration unit (Sartorius) and the content of uronic acids was determined according to the method of Knutson and Jeanes (1968). After 24 h of incubation the samples were mixed with 0.16 units of phosphodiesterase (EC 3.1.4.1) and 1.6 units of alkaline phosphatase (EC 3.1.3.1) (sample solution: pH = 9) and deproteinized after another 22 h by treatment with Centrisart I ultrafiltration unit. Finally, the reaction products of all samples were identified by thin layer chromatography performed at 25 °C on cellulose plates [solvent: acetic acid:pyridine:ethyl acetate:water (1:5:5:3)]. The reaction products were detected by liquid szintillation counting.

RESULTS

Enzymatic activities relevant for alginate biosynthesis were not detected by the optical assay with the exception of a small "phosphomannomutase" activity. Therefore we used a long time incubation for the demonstration of the (obviously very small) enzymatic activities.

Conversion of fructose-6-P into mannose-6-P (test for PMI).

Substance quantity (mM)

Fig. 1 Conversion of mannose-6-P into fructose-6-P

△ mannose-6-P — □ fructose-6-P — incubation assay

+ mannose-6-P — ✱ fructose-6-P — control assay

It was possible to detect an enzyme activity catalyzing the conversion of fructose-6-P into mannose-6-P in the crude protein extract (Fig. 1). The specific activity was 0.06 mU/mg. The state of equilibrium was on the site of fructose-6-P (equilibrium constant: 1.78). Therefore we used the reverse reaction.

Conversion of mannose-6-P into mannose-1-P (test for PMM)

We detected a small activity for the conversion of mannose-6-P into mannose-1-P by the optical assay (Fig. 2). In this case we also used the reverse reaction.

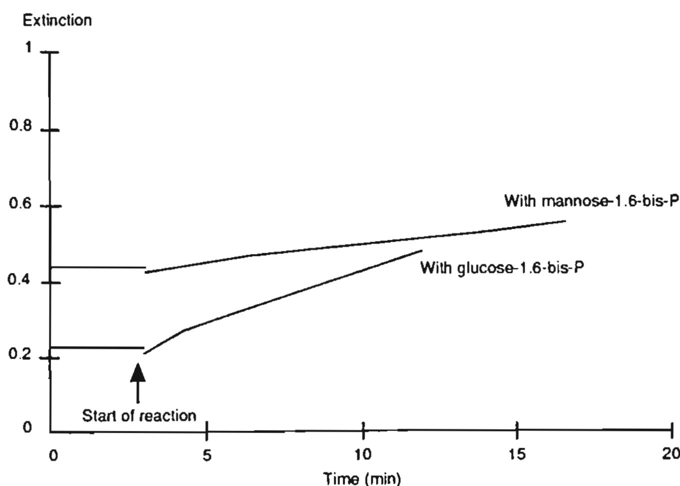


Fig. 2 Evidence for the conversion of mannose-1-P into mannose-6-P in the crude protein extract

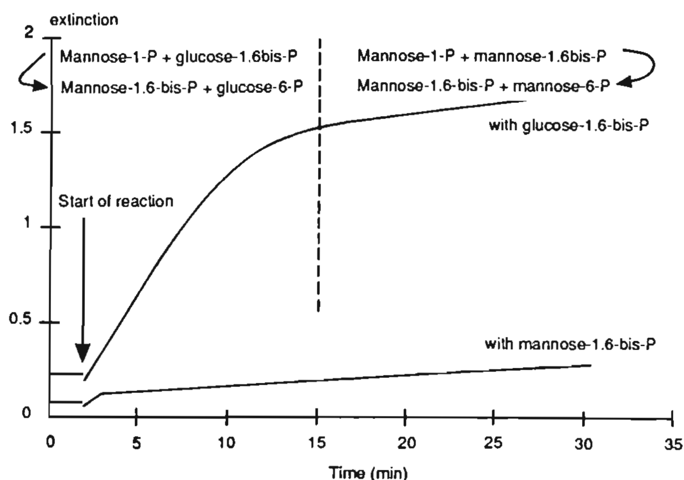


Fig. 3 Evidence for the conversion of mannose-1-P into mannose-6-P by commercial phosphoglucomutase from yeast

Commercial phosphoglucomutase from yeast also catalysed the conversion of mannose-1-P into mannose-6-P (Fig. 3). Therefore it is possible that the mutase activity in the crude protein extract is correlated with the phosphoglucomutase. A long incubation time was used for determining the specific activity (Fig. 4).
In the crude protein extract an enzyme activity catalyzing the conversion of mannose-6-P into mannose-1-P could be detected. The specific activity was 0.47 mU/mg.

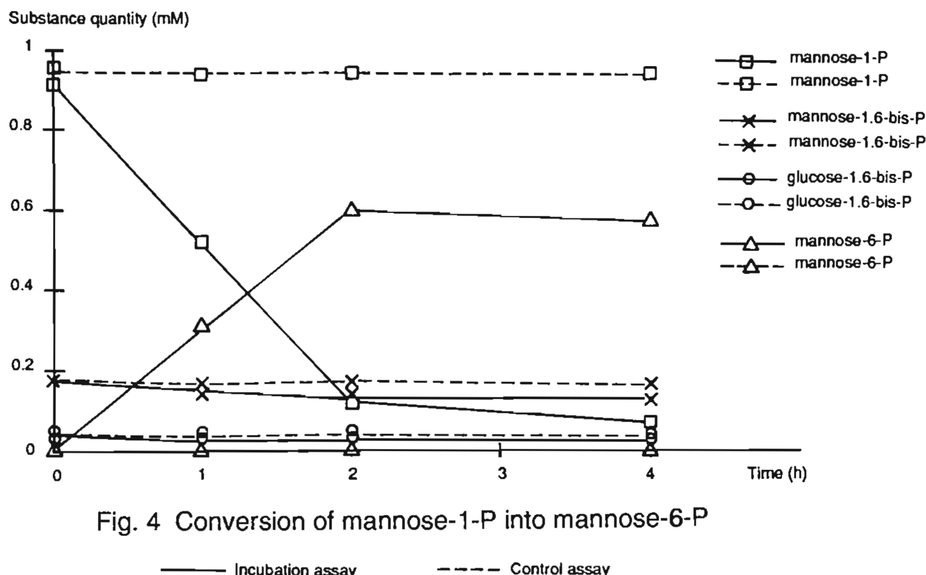


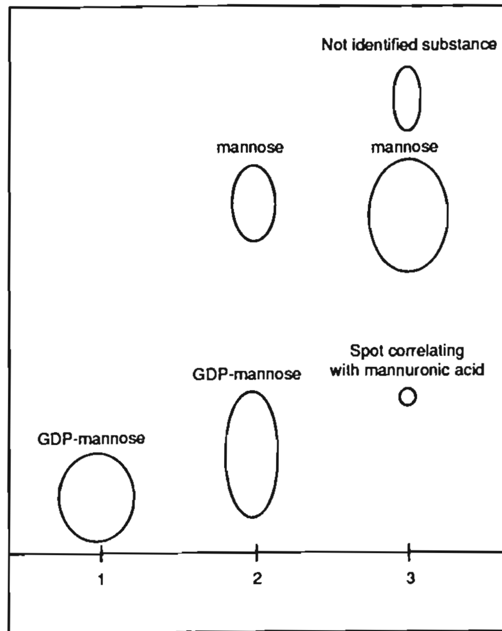
Fig. 4 Conversion of mannose-1-P into mannose-6-P

——— Incubation assay ---- Control assay

NAD-dependent oxidation of GDP-mannose in connection with formation of GDP-mannuronic acid (test for GMD).

Table 1 : Content of uronic acids

sample	incubation time	uronic acids [mg/l]
incubation	0 hour	0
assay	24 hour	97
incubation assay	0 hour	0
without NAD	24 hour	0
control	0 hour	0
assay	24 hour	4



1 : 0 h incubation time
2 : 0 h treatment with phosphodiesterase and alkaline phosphatase
3 : 22 h treatment with phosphodiesterase and alkaline phosphatase

Fig. 5 Thin layer chromatography analysis of GMD reaction products.

Whether the substance in the spot correlating with mannuronic acid represents mannuronic acid has to be shown by further studies.

The results indicate the presence of a NAD-dependent GDP-mannose dehydrogenase activity in the crude protein extract.

Direct oxidation of mannose in connection with formation of mannuronic acid.
We checked the direct oxidation by several methods:

- oxygen as electron acceptor (with and without presence of pyrroloquinoline quinone (PQQ) after long incubation time)
- NAD/NADP as electron acceptors (without presence of PQQ) by the optical assay
- dichlorphenol indophenol as electron acceptor (with presence of PQQ) by the colorimetric test

All these variants did not indicate an enzyme activity catalyzing the direct oxidation of mannose into mannuronic acid.

DISCUSSION

Our studies on the alginate biosynthesis by *Pseudomonas syringae* pv. *phaseolicola* strain 1321 indicate that this strain possesses the following enzyme activities: phosphomannose isomerase, mutase activity converting mannose-6-P into mannose-1-P and GDP-mannose dehydrogenase. However the specific activities were very small (< 0.1 mU/mg). The conceivable alternative biosynthetic pathway starting with direct oxidation of mannose into mannuronic acid did not seem to occur. Therefore it is possible that *P. s.* pv. *ph.* synthezises alginate during the first reaction steps analogous to the sequence described for *Pseudomonas aeruginosa.* However the mutase activity does not seem to be due to a "phosphomannomutase" but to the constitutive phosphoglucomutase. The future planned investigations are directed to additional problems of GMD and further enzymes in the alginate biosynthetic pathway.

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Polygalacturonase deficient strain of *Erwinia carotovora* ssp. *atroseptica* has attenuated virulence

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ABSTRACT

Low maceration rate of potato tubers caused by strain 22/8 of *Erwinia carotovora* ssp. *atroseptica* was related to low activity of polygalacturonase in macerated tissue. Similar levels of polygalacturonate lyase, protease and cellulase activities were detected in tissues macerated by that strain and the highly virulent strain 158. Pectic enzymes produced by both strains decomposed polygalacturonic acid and pectin with the same efficiency when assayed at pH above 8.0, however at pH 7.0, those produced by strain 22/8 generated larger amount of medium size oligogalacturonides. Necrotisation and better wound healing of the tubers infected with strain 22/8 resulted in termination of the rot 2-3 days after inoculation.

Low molecular compound(s) present in hot water extracts from the tubers suppressed polygalacturonase production by virulent strain 158 and induced production of two low molecular (20 kD) pectate lyases in strain 158.

INTRODUCTION

The role of pectic enzymes in pathogenicity and induction of plant defense reactions were presented by COLLMER & KEEN, 1986; KOTOUJANSKY, 1987; TSUYUMU et al. 1990. Molecular techniques have to be utilized for an understanding of pathogenicity and plant - bacteria communication. Strains that do not produce particular pectic enzymes give an

insight on this relationship. Preliminary observations on the correlation between virulence and pectic enzymes produced by strains 22/8 and 158 are presented below.

METHODS

Strains 158 and 22/8 were isolated in Bonin from decayed potato tubers and were maintained on slants of potato dextrose agar (Difco) neutralized to pH 7 with K-phosphate. Bacteria were propagated overnight on Luria broth, pelletted, rinsed, and a water suspension was used as inoculum at an initial concentration of 5×10^7cfu and ten-fold dilutions.

Polypectate medium was prepared as described by STACK et al. (1980) from apple polygalacturonic acid (Fluka) neutralized with KOH.

Extracts from tubers (Malwa cv.) were prepared by heating of peeled tuber cubes with 4 parts of water (w/v) for 20 min in a boiling water bath. Filtered liquid was stored for 2-3 days in refrigerator, after which the medium was filtered and neutralized with K-phosphate and autoclaved.

Freshly sliced desinfested tubers were inoculated with 10 μl portions of bacterial suspension. Fifty μl of inoculum were injected with yellow pipet tips into whole tubers. Infested tubers were incubated at 27 ^oC in a dew chamber. One day later soft rot of the slices was scored using a 0-5 grade scale (0 - no rot, 5 - severe rot). Maceration was evaluated on sectioned tubers as diameter of formed caves.

Enzymes in culture filtrates or fluid from macerated tissue were assayed with arsenomolybdate (polygalacturonase = PG) absorption at 234 nm (polygalacturonate lyase = PL), Azocoll solubilization (proteinase = Prt), formation of haloes on agarose gels amanded with pectin (pectinmethylesterase = PME) or carboxymethylcellulose (cellulase) when flooded with cetyltrimethylammonium bromide solution.

The molecular size of oligogalacturonides formed by pectic enzymes was examined by exclusion chromatography on Bio-Gel P-30 column.

PG and PL activities in SDS-polyacrylamide gel (6%) having incorporated 0.1% polypectate were visualized within the gel by displacing of the SDS from the complex with 1% Triton X-100 solution and repeated rinsing of the gel with buffers pH 5.5 (PG) or 8.3 (PL).

RESULTS AND DISCUSSION

Both strains were classified as *Erwinia carotovora* ssp. *atroseptica* on the basis of biochemical properties: reducing

sugars from sucrose (+); acid from methyl-cellulose (+); pits on CVP (+); growth on CPG + 5% NaCl (+); resistance to erythromycine; phosphatase activity (-); growth at 36° C on CPG. The growth rates and enzyme levels were similar for both strains except that significantly less PG (20%) was produced by strain 22/8 on polypectate medium and on the tubers. Maceration rates of the tubers by both strains is presented in Table 1.

Table 1.
Rotting of the tuber slices infected with two strains of *Erwinia carotovora* ssp. *atroseptica.*

	Strain 158				Strain 22/8			
cfu	$5x10^7$	$5x10^6$	$5x10^5$	$5x10^4$	$5x10^7$	$5x10^6$	$5x10^5$	$5x10^4$
Score	1.96	1.56	0.08	0.0	1.12	0.46	0.0	0.0

Mean for 5 cultivars with 5 tubers each; 0 - no soft rot; 1 - decolorization; 2 - 5 : lesions of 5, 10, 15, 20 mm diameter, respectively.

The same dominating basic forms of PL were present in the liquid from macerated tissue and in polypectate medium, however bacteria cultivated on tuber extracts produced additional PL enzymes (Fig.1).

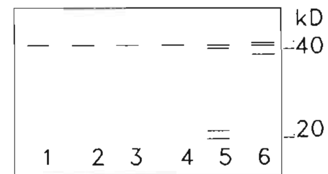

Fig 1. SDS-PGA gel electrophoresis of PL from: decayed tuber (1,2); pectate medium (3,4); and extracts (5,6); 1,3,5 - strain 158 ; 2,4,6 - strain 22/8;

Moreover, there was no PG at all in culture fluids from the extracts infested with strain 158 or 22/8 and no PG inhibitor was detected in them.
Extracts deprived of the sugars by previously growing bacteria on them, remained suppresive for PG production. Amandment of the potato extracts to the inoculum (strain 158) caused transient delay of the maceration and 50% decrease in PG activity in macerated tissue compared to tubers infected with water suspension of the same bacteria. All differences disappeared 15 hours later. More pronounced degradation of PGA by culture filtrate of strain 158 (PG + PL) was observed at pH 7.0 - close to physiological pH of the tuber juice (Fig. 2).

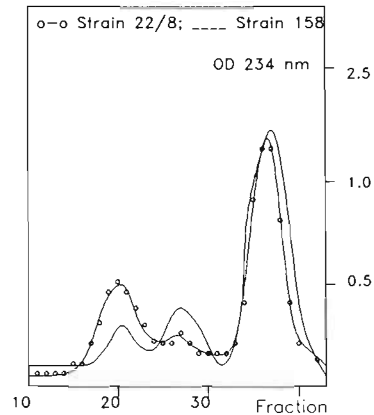

Fig 2. Exclusion chromatography of oligogalacturonides generated by pectic enzymes present in tubers macerated by Eca strain 158 (PG+PL) and 22/8 (PL)

These results implicate an involvement of PG in initiation of infection and better efficiency of the strains endowed both with PG and PL enzymes. This suggestion is only speculative and has to be confirmed experimentally with PG^- mutants of strain 158. According to Willis et al. (1987) PG is not an important factor of pathogenicity. Indeed, strain 22/8 attacked the tubers sucessfully and survived outside the laboratory.On the other hand, the intrinsic factor that only transiently suppress PG production, may promote defence reaction.

Low molecular PL's produced by the virulent strain 158 (Fig. 1) may suggest their significance for pathogenicity. However these forms do not occur in macerated tissue and in polypectate medium. This contradiction may be explained by specific expressions of plant inducible genes triggered by distinct sets of signalling compound(s) already present in the extract. Other signals are generated from cell walls by bacterial enzymes in the whole tissue. Sequentional and alternated synthesis of pectic enzymes by *Ecc* on polypectate medium and on tuber slices was reported by YANG et al.(1990). Several types of regulations of PL synthesis in *E. chrysanthemi* were described by REVERCHON et al. (1990).

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Purification and characterization of "alcaligin-E" a hydroxamate-type siderophore produced by *Alcaligenes eutrophus* CH 34

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ABSTRACT

Ten different strains of *Alcaligenes eutrophus* were analysed for their siderophore production in iron limiting medium. Six of them were found to respond to iron limitation by excreting siderophores in the culture media. These siderophores were detected using chrome azurol S. The siderophore of *A. eutrophus* CH34 (ATCC 43123) was purified by metal chelate affinity chromatography (MCAC). Paper chromatography and thin layer chromatography revealed that *A. eutrophus* CH34 produced only one siderophore. Chemical analysis showed that this siderophore is of the hydroxamate type. The molecular weight of this siderophore, designated alcaligin E, was determined bySephadex G-15 gel filtration and was shown to be about 1500 daltons.

INTRODUCTION

In iron depleted environments most bacteria synthesize and excrete strong iron chelators to scavenge iron from the environment. These ferric ion specific molecules are known as siderophores (NEILANDS, 1981). The siderophores excreted by microorganisms in the environment solubilize the iron from minerals or organic compounds (CROSA, 1989). Subsequently the iron is transported into the cell cytosol via a specific outer membrane receptor which recognizes the ferric-siderophore complex (AMES, 1986). The iron is released in the cell cytosol by reducing ferric to ferrous ion (JOHNSON *et al.*, 1991).

Alcaligenes eutrophus subsp. *metallotolerans*, a facultative, chemolithotrophic soil bacterium, is specially found in environments polluted with heavy metals. Ten strains of this species were analysed for siderophore production under iron limitting conditions. From one of the siderophore producing strains, *A. eutrophus* CH34, that was isolated from a

decantation basin of a zinc factory (MERGEAY *et al.*, 1978, MERGEAY *et al.*, 1985) and which has plasmid bound resistances for Ni^{2+}, Hg^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+} Cr^{6+}, Tl^{+} and Pb^{2+} ions, the siderophore was purified and examined in more detail.

In this paper we report the identification, purification and partial physico-chemical characterization of the siderophore produced by *A. eutrophus* CH34. This siderophore was designated alcaligin E.

MATERIALS AND METHODS.

Bacterial strains and culture media.

The *A. eutrophus* strains used in this study are listed in table 1, together with their siderophore synthesizing capacities. All A. *eutrophus* strains were kept and grown in Tris gluconate minimal salt medium (SCHLEGEL et al., 1961) containing the appropriate concentrations of either heavy metal, antibiotics or both. To examine the production of siderophores, cultures were grown in Tris gluconate medium with or without supplementation of iron. Cultures were incubated at 30°C.

Production and purification of the siderophore from *A. eutrophus* CH34.

A culture of strain AE1153, a siderophore overproducing mutant of CH34, was grown in Tris gluconate medium for 72 h to obtain maximal siderophore production. The cells were removed by centrifugation. Culture supernatant was then freeze-dried and dissolved in 20 mM Tris pH 7.5 to have it 60X concentrated.

A 34 X 2.5 cm column was packed with 200 ml Sephadex G-15. One ml concentrated culture supernatant was loaded onto the column. 20 mM Tris pH 7.5 was used as mobile phase. Fractions of 1 ml were collected and examined for the presence of siderophores using chrome azurol S (SCHWYN & NEILANDS. 1987) The siderophore positive fractions were pooled and subsequently freeze-dried. All siderophore positive fractions gave a specific absorption at 209 nm.

Metal chelate affinity chromatography (MCAC) was than employed for final purification. A column of 0.5 X13 cm was packed with metal chelate affinity chromatography medium (Pharmacia) using buffer “A” (50 mM Na acetate 200 mM NaCl pH 5.5) and charged with 3 ml of 50 mM FeCl3. The column was subsequently washed with two bed volumes of double distilled water to remove the non-bound iron and than equilibrated with buffer “A”. 50 mg freeze-dried material obtained after Sephadex G-15 column purification was dissolved in 200 µl of buffer “A” and loaded on the MCAC column. On this column, the siderophore binds specifically to the ferric ion. After having washed away the unbound impurities, the ferric-siderophore complex was eluted by 50 mM EDTA (pH 7.5). The siderophore containing fractions were identified by measuring the absorbance at 209 nm.

The siderophore positive fractions were pooled and subjected to gel filtration on Sephadex G-15 column as described before in order to remove the EDTA. As mobile phase 20 mM of (NH4)2 CO3 (pH. 7.5) was used.

Paper and thin layer chromatography.

Concentrated crude culture supernatant, partially purified (after Sephadex G-15) and purified siderophore were run on chromatography paper. The chromatography was carried out using as mobile phase (1-Butanol (butyl-alcohol) : glacial acetic acid: H^2O) in a ratio of 4: 1:1.

After chromatography, the paper was dried and sprayed with CAS solution to vizualize the siderophores.

A similar mobile phase and siderophore detection procedure was used in thin layer chromatography with Kieselgel 60 F aluminium plates.

Determination of the iron binding group(s) of alcaligin E.

To identify the iron binding group of alcaligin E, organic solvent extraction methods (COX & GRAHAM, 1979, PHILSON & LLINAS, 1989) and the methods of Csàky (1948) and Arnow (1937) were used according to these authors.

***Tn5-Tc* transposon mutagenesis of *A. eutrophus* CH34.**

Transposon mutagenesis of A. *eutrophus* CH34 was performed using the suicide delivery vector pSUP10141 (SIMON *et al.*, 1983). Matings between the donor strain *E.coli* S17-1 (pSUP10141), containing the transfer genes from RP4 integrated in its chromosome and the recipient strain CH34 were performed as described before (LEJEUNE *et al.*, 1983).Transconjugants were selected on iron limitted Tris gluconate plates containing Tc (20µ g/ml) and CAS to monitor their siderophore production.

Outer membrane protein preparation.

Outer membrane proteins were prepared as described before (CORNELIS *et al.*, 1989). The protein concentration in the preparation was determined by the method of Lowry (LOWRY *et al.*, 1951). Outer membrane proteins were analysed on 10% SDS-PAGE gels (LAEMMLI, 1970).

RESULTS AND DISCUSSION

When grown in iron depleted conditions, *A. eutrophus* CH34 produced siderophores which could be detected either in liqud medium or on plates using chrome azurol S. However, the addition of 2µM $FeCl_3$ to the minimal media completely suppressed this siderophore production. Nine other strains of A. *eutrophus* "subsp. *metallotolerans*" were also analysed for their siderophore production. For four strains, no siderophores were detected under our culture conditions (see table 1).

Table. 1
Strains of *A. europhus* tested for siderophore production using the chrome azurol S assay of Schwyn and Neilands (1987).

Strains	Siderophore production
CH34	+
AE104	+
DS185	+
SV661	+
AS39	+
AS168	+
AS2	-
AS24	-
ER107	-
ER122	-

In order to have a better understanding of the regulation of siderophore production at the molecular level in *A. eutrophus* CH34 and to see whether these siderophores were playing a role in the heavy metal resistance of this strain, we tried to obtain mutants affected in siderophore production or regulation. To do so Tn5-Tc mutagenesis was carried out using the suicide delivery vector pSUP10141. Three different classes of mutants were identified: biosynthetic mutants (affected in structural genes), not synthesizing siderophores in response to iron starvation; a constitutive siderophore producing mutant; and a third class of mutants which produce siderophores to much lesser extent as compared to the wild type strain CH34. The isolation of the constitutive mutant is an indication for negative regulation of siderophore production, as reported for the *fur* system of *E. coli* (HANTKE, 1981).
Since it is known from studies in *E. coli* (NEILANDS, 1982) and *Pseudomonas aeruginosa* (SOKOL, & WOODS., 1983., MARUGG *et al.*, 1989) that mutations affecting siderophore production and regulation might also effect the synthesis of IROMPS (iron regulated outer membrane proteins), we decided to identify these proteins in CH34 and to examine their synthesis in the CH34 siderophore mutants. Two iron regulated proteins of 78 and 80 Kda were identifed in strain CH34. In addition, 2mM Zn^{2+} could also induce the synthesis of these IROMPs, this even in the presence of 5µM iron, suggesting a second regulation of siderophore production by this metal. A similar observation was reported for *P.aeruginosa* 7NSK2 (HOFTE *et al.*, 1989). Subsequently, the IROMPs synthesis and regulation was examined in the siderophore mutants. The constitutive siderophore synthesizing mutant AE1153 was found to produce the 78 and 80 Kda IROMPs, even in the presence of 20µM $FeCl_3$. This indicates that the mutation did not only affect the regulation of the siderophore production but also of the IROMPs biosynthesis. Therefore it can be concluded that both are subjected to the same negative control mechanism. In contrast, the biosynthesis of the 78 and 80 KDa IROMPs in the siderophore negative mutant AE1093 and AE1152 was regulated as in the wild type strain CH34. However, in both siderophore negative mutants

examined, a protein of 88kda that is normaly constitutivelly expressed was shown to be absent. This indicates that except from the identified IROMPs, other proteins might play a role in siderophore secretion or uptake. The siderophore mutants will now be tested for their resistance to heavy metals.

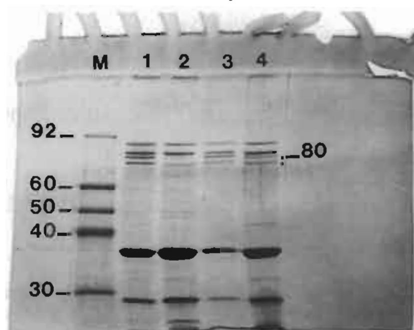

Fig. 1: Outer membrane protein profiles of *A.eutrophus* CH34 grown Tris gluconate medium supplemented as follows.
Lane. 1) Without Fe., 2) 20μM Fe., 3) 2mM Zn, 4) 20μM Fe 2mM Zn.

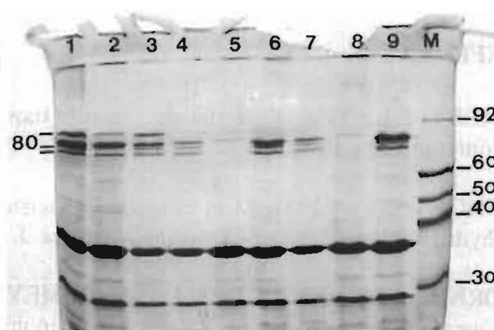

Fig. 2: Outer membrane protein profiles of *A. eutrophus* CH34 mutants grown in Tris medium supplemented as follows. Lane. AE1153. 1) Without Fe., 2) 20μM Fe., 3) 2mM Zn. AE1152. 4) Without Fe., 5) 20μM Fe., 6) 2mM Zn. AE1093. 7) Without Fe., 8) 20μM Fe., 9) 2mM Zn. M) Protein marker.

To examine how many siderophores were produced by *A. eutrophus* CH34, paper chromatography and thin layer chromatography were performed on culture supernatant of this strain. After staining with the CAS solution, only one spot was observed (results not shown) indicating that only one siderophore is produced by *A. eutrophus* CH34. This siderophore was designated alcaligin E. Subsequently, the physico-chemical properties of this siderophore were studied. The iron binding group(s) of alcaligin E was determined using organic solvent extraction methods (COX & GRAPHAM, 1979., PHILSON & LLINAS, 1989.) and was found to be of the hydroxamate type. This result was confirmed by the method of Csàky. The method of Arnow,that specifically detects catechol groups, gave a negative result. The molecular weight of alcaligin E was dtermined by gel filtration using Sephadex G-15. Vitamin B12 (Mw. 1355 daltons) and insuline (Mw. 3400 daltons) were used as molecular weight markers. The molecular weight of alcaligin E was determined to be about 1500 daltons.

The next step was the purification of alcaligin E. In order to have higher siderophore concentration in the culture supernatant, the constitutive siderophore producing mutant AE1153 was used. Siderophore purification was achieved by coupling two chromatographic techniques, gel filtration by Sephadex G-15 and metal chelate affinity chromatography (MCAC). The specific affinity of siderophores to ferric ion was used for their final purification by binding them to ferric ion that was immobilized to the imminodiacetic acid group of the MCAC column. After the binding of alcaligin E to ferric ion no leakage of siderophores from the column was observed. After the recovery of alcaligin E from the column usig EDTA and a last purification by gel filtration, a highly purified

siderophore preparation was obtained. This will now be used for FAB-mass spectrometry and NMR studies in order to elucidate the structure of alcaligin E.

In the future we will also test the usefullnes of this method for the purification of other siderophores.

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Virulence of isolates of *Pseudomonas solanacearum* from worldwide sources on resistant and susceptible tomato cultivars

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ABSTRACT

Interactions between pathogenic strains of *Pseudomonas solanacearum* and tomato cultivars (with and without resistance genes from different sources) were compared under controlled environmental conditions. The pathogen strains varied in ability to cause typical bacterial wilt symptoms on each cv. One strain (a biovar 2 isolated from potato in Pangalengan, Indonesia in 1991) caused wilt within 14 days on all cvs. (Venus, Saturn, UPCA-1169, Intan, Ratna, Scorpio, Kewalo, CRA-66, Caraibo and Rodade), all of which have been reported as resistant in one or more countries. The virulence of each strain was related to its aggressiveness on the susceptible cultivar Moneymaker; the least aggressive strains showing compatible interactions with the least number of cvs. Although some strains failed to induce wilting within the 28 day observation period, analysis of the homogenate of stem sections by indirect ELISA (using polyclonal antisera) often detected high populations of the pathogen (10^5-10^9 cfu per ml) in the stem base and sometimes mid-way up the stem. Colonisation of this type was usually accompanied by brown discolouration or necrosis of xylem tissues in the same region. The bacterium was always detected at the apex of wilted plants. Only cultivars in which the pathogen could not be detected were considered resistant, otherwise they were termed susceptible or tolerant depending on whether they wilted or not. The only incompatible interaction observed between an aggressive pathogen strain and a resistant cv. was that between strain R38 (race 1/biovar 1 isolated from tomato in USA in 1953) and the cv. Kewalo from Hawaii.

INTRODUCTION

Pseudomonas solanacearum, the causal agent of bacterial wilt of a wide range of host plants, is a highly variable bacterial plant pathogen. Informal groupings at the infrasubspecific level have been used to divide the species into five races and five biovars (Hayward, 1991) which reflect host range and geographical distribution of the strains to some degree. However, there exists a high degree of heterogeneity within these groupings, particularly regarding the pathogenicity of strains of the bacterium on different hosts.

There are numerous reports worldwide on the selection of cultivars of tomato and other crop hosts with resistance to *P. solanacearum* (Eden-Green &

Elphinstone, 1993). Resistance, however, appears to be site-specific i.e. the selection of a resistant cultivar in one location does not guarantee its resistance and selection in another. It is currently unclear to what extent this phenomenon is due to the evolution and distribution of strains of the pathogen differing in virulence, or to the complicating influences of environmental and other biotic factors (temperature, moisture availability, soil type, light intensity, photoperiod, interaction with antagonistic or synergistic organisms etc.) on the host-pathogen interaction (Hayward, 1991). This report summarises research, conducted in 1991 at Rothamsted Experimental Station, to study the interaction, under controlled environmental conditions, between diverse strains of *P. solanacearum* and cultivars of tomato which have been used as sources of bacterial wilt resistance in breeding programmes.

MATERIALS AND METHODS

Bacterial isolates: Isolates of *P. solanacearum* were stored at -80 °C in a glycerol-peptone solution and cultured on casamino-peptone-glucose (CPG) agar (Kelman, 1954) at 30 °C. Aqueous suspensions of inoculum were prepared by washing 24 hr solid-agar cultures with sterile distilled water and the concentration was standardised by measuring optical density spectrophotometrically at 650 nm (Prior *et al*., 1990). The origin of isolates used is given in Table 1.

Table 1: Isolates of P. solanacearum used in this study

Code	Other code	BIOVAR	RACE	HOST	Country	Year	Source
R038	UW19, K-60	1	1	Tomato	USA	1953	Kelman
R279	UW151	4	1	Ginger	Australia	1965	Hayward
R283	UW167	1	2	Banana	Costa Rica	1958	Sequeira
R586	CIP314	N2	?	Tomato	Peru	1989	Aley
R653	PAN1	2	3	Potato	Indonesia	1991	Elphinstone
R659	SUB3	3	1	Tomato	Indonesia	1991	Elphinstone
R664	PSS4	3	1	Tomato	Taiwan	1988	Hartman

Tomato cultivars: Cultivars with different sources of resistance to *P. solanacearum* (Table 2), selected in different geographical locations worldwide (Eden-Green and Elphinstone, 1993), were obtained from Dr R Opena at the Asian Vegetable Research and Development Center, Taiwan. The cv. Moneymaker, supplied by Kings of Kelevedon, UK, was used throughout as a susceptible check.

Table 2: Tomato cultivars used in this study

Cultivar (AVRDC Code)	Resistance source	Reporting countries
Venus (L-96)	*L. pimpinelifolium* (PI 129080)	IDA MAL SL TAI USA MAR
Saturn (L-96)	*L. pimpinelifolium* (PI 129080)	SL TAI USA MAR
UPCA-1169 (L-12)	Venus, CA-64-1169	MAL PHI
VC 48-121 (L-33)	UPCA-1169	IDA PHI SIN
VC 11-1UG (L-21)	UPCA-1169	IND IDA MAL PHI TAI REU SEY
Kewalo (L-274)	*L. pimpinelifolium* (PI 127805A)	USA
CRA-66 (L-3970)	*L. esculentum* var. *cerasiforme*	IND USA GUA MAR
CARAIBO (BL-341)	CRA-66	REU GUA MAR
Rodade (BL-437)	BW-2	SA PNG AUS

Countries: AUStralia, GUAdeloupe, INDia, InDonesiA, MALaysia, MARtinique, Papua New Guinea, PHIlippines, REUnion, SEYchelles, SINgapore, South Africa, Sri Lanka, TAIwan, USA.

Inoculation procedures: A variation of the inoculation method of Winstead and Kelman (1952) was used. Seedlings were germinated and, after 1 week, transferred to plastic pots of 70 mm diameter containing sterilised compost (Croxden Prescription Compost, Freehay, Cheadle, Stoke on Trent, UK). The seedlings were grown on in Wisconsin tanks with constant soil and air temperatures of 28 and 25 °C respectively, a relative humidity of 70%, a daylength of 16 hrs and light intensity of 13,000 lux. After a further 14 days the seedlings were inoculated by cutting the roots through the compost to one side of the plant with a sterile scalpel and adding 20 ml of an aqueous inoculum suspension containing 1×10^7 cfu (colony-forming units) per ml. Five seedlings per cultivar were inoculated with each strain and control plants of each cultivar were inoculated with sterile distilled water.

Disease assessments: The relative agressiveness of each strain was compared on the susceptible cultivar Moneymaker by estimating the rate of symptom development weekly for 4 weeks on a 1-5 scale of increasing severity (Prior *et al.*, 1990). Disease development, following inoculation of the various cultivars with each pathogen strain, was assessed similarly. In addition, the degree of longitudinal colonisation of the stem was determined using an indirect ELISA procedure (Robinson, 1993). Homogenates of 1 cm stem sections from the base, middle and apex of each inoculated plant were tested for the presence of the pathogen using polyclonal antisera produced to strain R283 of *P. solanacearum* (Robinson and Eden-Green, unpublished).

RESULTS AND DISCUSSION

Considerable variation was observed in both the aggressiveness of strains on the susceptible cultivar Moneymaker (Fig. 1) and their ability to colonise and induce wilting on the resistant cultivars (Table 3). Although some strains failed to induce wilting within the 28-day observation period, dissection of the stems often revealed discolouration of vascular tissues (often leading to necrosis and lower leaf drop but not to wilting or plant death). These secondary symptoms were sometimes accompanied by stunted growth and/or chlorosis and necrosis (usually of the lower leaves). One strain (R653) caused typical wilting symptoms on all of the cultivars within one week after inoculation; a surprising result given the identity of this strain (biovar 2 isolated from potato in Pangalengan, Indonesia in 1991).

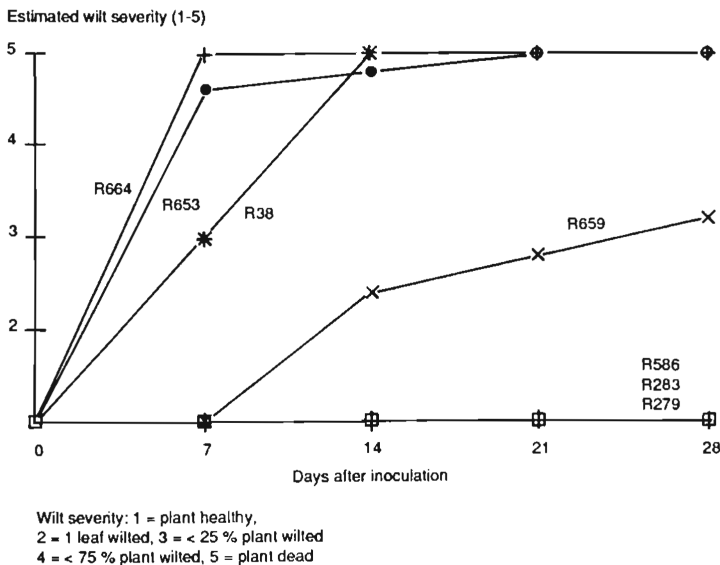

Fig.1: Aggressiveness of diverse isolates of *P. solanacearum* on the susceptible cultivar Moneymaker under controlled environmental conditions.

Analysis of the homogenate of stem portions by ELISA showed that wilted plants were always found to yield high pathogen populations (10^5 - 10^9 cfu per ml) from the basal, middle and apical regions of the stem. Cultivars which developed typical wilt symptoms leading to death of the plant were considered susceptible. Secondary symptoms were always associated with high populations of the pathogen in the stem base. In 75 plants of cultivars which demonstrated secondary symptoms, the pathogen was also detected in 31% and 5% of stem sections from the middle and apical regions respectively. Cultivars which remained symptomless

and in which the pathogen was not detected were considered resistant under the experimental conditions employed. Cultivars which survived with secondary or no symptoms but which supported high pathogen populations internally were termed tolerant.

Table 3: Resistance, tolerance and susceptibility of tomato cultivars to diverse isolates of *P. solanacearum* under controlled environmental conditions.

Cultivar	Pathogen strain (Rothamsted code)						
	653	664	038	659	586	279	283
CRA-66	+	+	+	-	(-)	(-)	-
VC48-121	+	+	+	(-)	(-)	-	-
Venus	+	+	+	(-)	-	-	(-)
Rodade	+	+	(-)	(-)	+	(-)	-
UPCA-1169	+	(-)	(-)	-	(-)	-	-
Caraibo	+	(-)	(-)	-	-	-	-
Kewalo	+	(-)	-	-	+	-	-
Saturn	+	(-)	(-)	-	(-)	-	-
Moneymaker	+	+	+	+	(-)	(-)	(-)

\+ Susceptible (at least one out of 5 inoculated plants wilted).
(-) Tolerant (no wilt but > 10^5 cfu per ml of pathogen detected in homogenate of stem base section).
\- Resistant (no wilt and pathogen not detected in stem base).

No resistant reactions were observed on the cultivar Moneymaker, although not all strains induced wilting under the experimental conditions employed. Virulence of the various strains on the range of cultivars was related to their aggressiveness on the susceptible cultivar. The less aggressive strains showed low virulence on the range of cultivars tested and *vice versa*. Where cultivar resistance or tolerance was observed, it was not general for all strains. Differential interactions which occured between particular strains and cultivars will be further studied to determine their stability under a range of inoculum concentrations and soil temperatures. Testing of a larger number of strains is also planned to allow grouping of strains with similar virulence.

The only totally incompatible interaction involving a highly aggressive strain was that between the type strain R38 (Biovar I, race 1 from USA) and cv. L-274 ("Kewalo" from Hawaii). Also of particular interest was the susceptibility of this cultivar (as well as cv. "Rodade") to the biovar N2 strain R586 despite the fact that this strain caused only secondary symptoms on the check cultivar Moneymaker.

Strains R664 (Biovar 3; used at AVRDC in Taiwan for resistance screening) and R659 (Biovar 3; isolated from an infested nursery at Subang, Indonesia in 1991) were compared to determine whether differences in virulence between the two strains could explain why resistant cultivars selected at AVRDC are often susceptible when tested in the Indonesian nursery. The results (Table *) did not support this hypothesis since the Indonesian strain was much less virulent than the Taiwanese strain. However, it is interesting that the most virulent strain (R653) was also from Indonesia. The nursery at Subang had been artificially inoculated. Further testing is needed to confirm the virulence of the isolate used for inoculation and also to determine whether other, more virulent, strains are naturally present in the nursery.

Screening methods currently used to compare levels of resistance to *P. solanacearum* rely on measurements of incidence or severity of wilting symptoms. The results presented above suggest that the degree of multiplication and colonisation by the pathogen *in planta* are also important criterea, particularly if the selection of tolerant cultivars which carry latent infection is to be avoided. Similar findings have been presented by Prior *et al.* (this proceedings).

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Induced resistance to *Pseudomonas syringae* pv. *tabaci* transmitted from tobacco leaf to plants regenerated *in vitro*[1]

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Abstract

Tobacco plants cv. White Burley were regenerated up to the 10-14 leaf stage from leaf half tissue treated 48 h earlier with protein-lipopolysaccharide complexes (pr-LPS). Control plants were regenerated from opposite leaf halves infiltrated with water. The pr-LPS pretreatment induced localized protection to *Pseudomonas syringae* pv. *aptata (P.s.a.)*.

Leaves from regenerated tobacco plants were subjected to two challenge inoculations with *Pseudomonas syringae* pv.*tabaci (P.s.t.)* to check the presence of induced resistance: intercellular infiltration (5×10^6 CFU·ml^{-1}) and spraying with a suspension (10^8 CFU·ml^{-1}) in a moist chamber. Intercellular growth of *P.s.t.* after 48 h was less than 52% as compared to the control leaves. Twenty-one days after the spray inoculations, only the distinct necrotic areas (% leaf area) in the leaves of tobacco plants regenerated from leaf tissue treated with pr-LPS were significantly lower (65%) than those of the control leaves. Disease intensity (total affected area) was 68.2% as compared to the control.

This suggests that localized induced resistance may be directly transmitted from tissue where primary induction took place.

Key words: Induced resistance, protein-lipopolysaccharide complexes, plant regeneration, tobacco.

Introduction

Localized resistance to *Pseudomonas syringae* pv.*tabaci* (*P.s.t.*) can be induced by treating tobacco leaves 48 h earlier with protein-lipopolysaccharide complexes (Mazzucchi *et al.*, 1979). It consists of the prevention or delay of disease symptoms, reduction in their intensity and, at the same time, the inhibition or slowing down of endophytic bacterial growth. It is uncertain whether induced resistance can be transmitted and maintained through plant vegetative propagation. In tobacco there is some controversy as to whether induced systemic resistance to *Peronospora tabacina* can be transmitted to plants regenerated *via* callus from plant tissue where primary induction has taken place (Lucas *et al.*, 1985; Kuc',1987).

The aim of this study was to test the hypothesis that localized induced resistance to *P.s.t.* in tobacco leaves, obtained by pr-LPS treatment, can be maintained through shoot regeneration from leaf tissue.

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Materials and methods

Cultures. The strains of *P.s.t.* NCPPB 1918 and *P.s.a.* NCPPB 2664 were grown on YDC agar slants at 27°C for 24 h .

Turbidity. This was measured spectrophotometrically at 660 nm.

Plate counting. The Mazzucchi and Comelli technique was used (Mazzucchi *et al.*, 1979).

pr-LPS complexes. These were extracted and purified using the method of Mazzucchi *et al.* (1988).

Plant regeneration. Pieces, 0.5-1 cm^2, were taken from leaf halves of tobacco plants cv. White Burley infiltrated 48 h earlier with pr-LPS complexes (250 $\mu g \cdot ml^{-1}$) and kept in a climatic chamber at 25°C with a photoperiod of 14 h . Control pieces were taken from the opposite leaf halves preinfiltrated with water. The pieces of leaf tissue were washed, surface sterilized (immersion for 30-60 sec in ethanol and then in sodium hypochloride 1% for 30 min). After 5 washings in sterile distilled water, the pieces were transferred to Petri dishes containing 20 ml of MS medium (0.7% agar) (Murashige and Skoog, 1962) with addition of benzyladenine (1 $mg \cdot l^{-1}$). Plates were kept in a climatic cell at 24°C with a 16 h photoperiod at 4000 lux. After one month, the shoots regenerated as greenspots from the edge of the leaf pieces were individually transplanted to MS medium in Magenta GA7 vessels (Sigma) and kept under the same conditions. The rooted plantlets were transplanted to individual pots (Ø 10 cm) containing peat and sterile perlite, kept for one week with high humidity inside polyethylene bags, and then grown in the greenhouse for two months at 24-28°C with 95% R.H. up to the 10-14 leaf stage. The plants regenerated from leaf tissue treated 48 h earlier with pr-LPS complexes were the treated variants; those regenerated from leaf tissue treated 48 h earlier with water were the control variants.

Endophytic growth. The leaf halves intercellularly infiltrated with *P.s.t.* 24 h earlier were washed in running water, distilled water and then blot dried. Each sample consisted of 10 discs (Ø 10 mm) randomly collected from the interveinal areas of one leaf half and ground in a mortar for plate counting (Mazzucchi *et al.*, 1982).

Challenge inoculations. Known doses of *P.s.t.* were intercellularly infiltrated or sprayed on the leaves.

The interveinal areas on the more mature leaves of the treated and control variants were infiltrated with $5x10^6$ $CFU \cdot ml^{-1}$. After infiltration the leaves were washed with distilled water and the plants kept at 25°C with a photoperiod of 14 h and 70-90 % R.H. for 24 h before assessing endophytic growth.

Treated and control variants were kept in a moist chamber at 25°C inside polyethilene bags for 24 h and then all leaves were sprayed with 10^8 $CFU \cdot ml^{-1}$. After a further 24 h in the moist chamber, the bags were removed and the plants were kept in a greenhouse at 25°C with 90-95% R.H. After 21 days the leaves were stripped from each plant from top down to the one with incipient senescence,

excluding the apical leaflets. The diameter of the necrotic spots, the chlorotic haloes, the maximum length and width were measured from each leaf. A total of 192 leaves were assessed. In each leaf five parameters were recorded: area, spot number, distinct and coalesced chlorotic haloes, distinct and coalesced necrotic areas, total area affected.

Statistical analysis This was performed on angular values of percentage data, by using a CoStat software. Control and treated variants were analyzed to randomized complete block design with 96 replications: 8 leaves per plant, 12 plants for each variant.

Results

Confluent necrosis was not induced by infiltration of *P.s.a.* in the basal interveinal areas of leaf halves of plants grown from seeds treated 48 h earlier with pr-LPS complexes, but it was induced in those pretreated with water. All the pieces collected in the meantime from the corresponding leaf halves and transplanted to the MS medium were therefore suitable for the regeneration of plants from sensitized and control tissue.

The pieces of leaf halves treated 48 h earlier with pr-LPS complexes showed slight chlorosis at the time of collection, but they had a minor development as compared to the control only in the first days following transplant. After one month several shoot primordia seen as dark green spots, developed around the edges of the treated and control tissue discs.

Rooting and development of the plantlets during regeneration and the final appearance of the treated variants was indistinguishable from that of the control variants.

In the treated variants, 24 h after intercellular infiltration with *P.s.t.*, all the interveinal areas were asymptomatic. The mean numbers of *P.s.t.* cell counts in the leaves and control variants were 3.2×10^5 and 6.15×10^5 respectively. The difference between the two population levels was highly significant ($P < 0.001$). The bacterial population in the treated variants was only 52% of that in the control; 3 days after infiltration, however, the infiltrated leaf tissue was necrotic in both cases.

TABLE 1. Assessment of disease intensity according to five parameters (affected leaf area, %) 21 days after challenge inoculation with *Pseudomonas syringae* pv. *tabaci*.

Regenerated plants	Distinct chlorotic area	Coalesced chlorotic area	Distinct necrotic area	Coalesced necrotic area	Total affected area
CONTROL	1.40	0.11	0.37	0.08	1.89
TREATED	1.06	0.09	0.13	0.08	1.29
F-TEST	1.68	0.01	4.80*	0.04	2.51

* Significant at $P < 0.05$

In the 10-14 leaf regenerated plants, 21 days after challenge inoculation by spraying, numerous necrotic areolae surrounded by marked chlorotic haloes with sharp borders were visible on both variants. There was a difference in 4 out of 5 parameters adopted to evaluate disease intensity in the control and treated variants (Table 1). No difference was found as regards the percentage of confluent necrotic areas which did however represent only a minority (5.8% and 2.1% of the necrotic areas of the control and treated variants respectively) of the spots caused by the experimental inoculation. Statistical analysis of the angular values showed that only the diameters of the distinct necrotic areas were significantly different ($P < 0.05$), the mean values were 0.37% and 0.13% for the control and treated variants respectively. However, the total affected leaf area in the treated variants was 68.25% that in the control.

Discussion

The tobacco plants of the treated variants were regenerated from leaf tissue treated 48 h earlier with pr-LPS complexes to prevent confluent necrosis caused by *P.s.a.*, that is constantly associated with localized induced resistance to *P.s.t.* (Mazzucchi *et al.*,1979). In the leaves of the treated variants the endophytic *P.s.t.* population 24 h after inoculation was 52 % of the control. Obviously, the leaves of plants regenerated from explants of leaf tissue pretreated with pr-LPS complexes were less susceptible to *P.s.t.* colonization. There is also a minor endophytic growth of *P.s.t.* 24 h after infiltration in pr-LPS pretreated leaves of plants grown from seeds (Mazzucchi *et al.*, 1982); in this case, however, the population level is 12.5 % of the control. This indicates that leaf tissue resistance to bacterial colonization following primary induction is stronger than that in plants regenerated from the same tissue.

The intensity of the disease in the treated variants, 21 days after challenge inoculation with *P.s.t.*, was significantly lower than in the control variants when assessed as the percentage of individual necrotic areas, but not on the basis of coalesced chlorotic or necrotic areas or individual chlorotic haloes. The smaller diameter of the distinct necroses indicates less tissue colonization by bacteria and this is in agreement with a minor endophytic growth of *P.s.t.* within 24 h after inoculation. The non-significance of the difference between confluent necroses may be explained by taking into account that the closest necrotic lesions interfered somehow with each other when they coalesced.

In conclusion, in the leaves of plants regenerated from explants of leaf tissue pretreated with pr-LPS complexes the resistance induced to *P.s.t.* is expressed as the inhibition of bacterial growth in the first 24 h and in a minor leaf area affected by necrotic spots 21 days after the challenge inoculation.

These results indicate that induced resistance to *P.s.t.* can be transmitted at a certain extent to plants directly regenerated from tissue in which primary induction has taken place. The induced resistance transmitted to the regenerated plants appears as a kind of horizontal resistance (Fry, 1982). Our conclusions are in agreement with those of Kuc' (1987), but not with those of Lucas *et al.* (1985). The models, however, are not strictly comparable since these cases refer to the transmission of systemic and not localized induced resistance. No transmission of induced resistance to *P.s.t.* was found, in tobacco plants indirectly regenerated *via* callus in the presence of heat-killed cells of *P.s.a.* in MS medium or infiltrated under vacuum in the explants, with a 0.1% (v/v) concentration (unpublished data).

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Characteristics of toxins produced *Pseudomonas syringae* pv. *atrofaciens*

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ABSTRACT

Pseudomonas syringae pv. *atrofaciens* causing disease of wheat produces toxins. HPLC analysis of the toxin preparation resolved nine peaks. Six main peaks corresponded to syringomycin (or similar toxin), three hydrophobic peaks with weak antifungal activity resembled lipodepsipeptides of *P. s.* pv. *syringae*. Amino acid analysis of total preparation of all nine peaks revealed the presence of Ala, Val, Dab, Ser, Arg, Leu, Gly, Pro, Asp, Thr, Tyr, Phe. All tested 50 strains of yeasts belonging to genera *Bullera*, *Cryptococcus*, *Metschnikowia*, *Sporodiobolus*, *Rhodosporidium*, *Rhodotorula* were sensitive to *P. s.* pv. *atrofaciens* strains 1007, IIPGR V8, IIPGR VI. Yeasts *cryptococcus hungaricus* strain BKM Y-1600, *Bullera grandispora* BKM Y-2662, *Cryptococcus dimennae* BKM Y-1644, *Rhodosporidium diobovatum* BKM Y-764 are useful as biotest for indication of toxin activity.

KEYWORDS

Toxins, *Pseudomonas syringae* pv. *atrofaciens*, yeasts.

INTRODUCTION

The phytopathogenic bacterium *Pseudomonas s.* pv. *atrofaciens* causes disease of cereals (VON KIETZELL & RUDOLPH, 1991 ; KOROLEVA & SIDORENKO, 1978). As for *P. s.* pv. *syringae* (GROSS, 1991) it is known that title bacterium produces a necrosis inducing toxin (VASSILEV *et al.*, 1991 ; ANISIMOVA *et al.*, 1991). The object of this study was isolation and characterization of toxins from *P. s.* pv. *atrofaciens*.

MATERIALS AND METHODS

P. syringae pv. *atrofaciens* strain 1007 (from wheat, collection of Institute of Microbiology and Virology, Ukraine) strains IIPGR V8 and IIPGV VI (from wheat, collection of Institute of Introduction and Plant Genetic Resources, Bulgaria) were grown on potato-glucose broth of agar supplemented with 0.4% casamino acids. Fifty strains of yeasts of the genera *Bullera*, *Cryptococcus*, *Metschnikowia*, *Sporodiobolus*, *Rhodosporidium* and *Rhodotorula* were obtained from Russian Collection of Micro-organisms.

Geotrichum candidum was used as biotest for study of toxin production by *P. syringae* pv. *atrofaciens*.

Toxin preparation by means of treatment of the bacterial culture with acetone, followed by n-butanol and carboxymethyl cellulose CM-52 ion-exchange chromatography (GROSS & DE VAY, 1977) was obtained from *P. s.* pv. *atrofaciens* 1007.

Toxin sample was investigated by HPLC LKB system equipped with reverse-phase column Silasorb C18, 3x250 mm (flow rate - 0,6 ml/min ; column temperature - +50°C, detection wave - 214 nm). Elution was performed by mixing solvent A (0.2% trifluoroacetic acid in water) and solvent B (10.1% trifluoroacetic acid in acetonitrile/isopropanol, 4/1, v/v). Fractions were collected at Supertac LKB - 2211 collector and then used for the *G. candidum* bioassay.

Amino acid analyses were carried out with Biotronic LC6000E amino acid analyzer by standard method. 2,4-diaminobutyric acid was tested by thinlayer chromatography (BOUSFIELD *et al.*, 1985).

RESULTS AND DISCUSSION

Chromatographic analysis of toxin preparation resolved six main peaks.

So we assume the occurrence of several forms of toxin isolated from *P. s.* pv. *atrofaciens* 1007 as it was reported (BALLIO *et al.*, 1988) for syringomycin or syringotoxin from isolates of *P. s.* pv. *syringae*.

But HPLC fractionation revealed three additional hydrophobic peaks which possessed weak antifungal activity. Recently A. BALLIO with co-workers (BALLIO *et al.*, 1991 ; personal communication) isolated from investigated strains of *P. s.* pv. *syringae* new phytotoxic hydrophobic metabolites called syringopeptins. So we proposed that three hydrophobic peaks are syringopeptins.

Amino acid analysis of all nine peaks indicated the composition : 2,4-diaminobutyric acid, threonine, aspartic acid, leucine, proline, alanine, valine, phenylalanine, arginine, serine, glycine, tyrosine.

For characterization of toxins of *P. s.* pv. *atrofaciens* strains we studied their effect on growth of yeasts that colonized the phyllosphere of different plants (Table 1). It was found that the yeasts *Cryptococcus hungaricus* strain BKM Y-1600, *Bullera grandispora* BKM Y-2662, *Cryptococcus dimennae* BKM Y-1644, *Rhodosporidium diobovatum* BKM Y-764 were most sensitive to toxins (inhibition zone more than 50 mm). These strains are useful as biotest for indication of toxin activity.

Table 1 :
Inhibition of the yeasts growth by *Pseudomonas syringae* pv. *atrofaciens* strains 1007, IIPGR V8, IIPGR VI

Yeast genera	Diameter of inhibition zone (mm)
Bullera (5,5)*	18
Cryptococcus (5,5)	13-30
Metschnikowia (3,5)	11-20
Sporodiobolus (5,5)	13-22
Rhodosporidium (3,9)	10-50
Rhodotorula (9,21)	6-20

* Number of tested species and strains.

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Syringotoxin action on the membrane level

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ABSTRACT

Planar lipid bilayers were used to study the influence of syringotoxin on the electrical conductance of lipid membranes. The results of our experiments show that the membrane conductance exponentially depends on the toxin concentration in the adjacent aqueous solutions. The increased membrane conductance is caused by ion channels formed by syringotoxin in the bilayer. The conductance increase of these channels is well defined, highly uniform, and directly related to the specific conductance of the respective aqueous solutions used. Using a trans-membrane salt gradient the channels were shown to behave anion selective. The selectivity is caused by a positive net charge in the channel structure which additionally plays an important role in the process of toxin incorporation into the lipid membrane.

Our results suggest that the phytotoxic activity of syringotoxin is linked with its ability to induce ion conductive channels able to depolarize affected membranes.

KEYWORDS

Syringotoxin, ion channels, lipid bilayer membranes, channel selectivity, pH-dependence.

INTRODUCTION

Syringotoxin (ST) and syringomycin are phytotoxins produced by bacteria of the species *Pseudomonas syringae* pv. *syringae* whereby strains Ps 268 is the one producing syringotoxin (GROSS & DE VAY, 1977a). This bacterial species is known to cause diseases in stone and citrus fruit trees as well as in other cultural plants in nearly all growth areas of the world (GROSS & DE VAY, 1977b). As shown by GROSS & DE VAY (1982) these toxins induce almost the same symptoms as the relevant bacterial species. Experiments on maize mitochondria revealed that both toxins display an ionophoretic activity whereby ST was found as being almost twice as active as syringomycin (SURICO & DE VAY, 1982).

Based on these results we tried to find out whether or not syringotoxin could affect the permeability behaviour of the lipid part of biomembranes. For this purpose planar lipid bilayers were used as model membranes on which conductance measurements were performed.

MATERIALS AND METHODS

Bimolecular lipid membranes were prepared according to MUELLER *et al.* (1962) from soybean lecithin (SERVA) dissolved in n-decane (20 mg/ml). This membrane forming solution was applied by micropipette to a hole (0.4 mm in diameter) in a teflon septum separating the aqueous solutions of cis- and trans compartment. Fig. 1 shows schematically the experimental set-up. A function generator providing constant and triangular voltages was connected to the cis-compartment and a pikoampere meter (KEITHLEY, model 619, USA) to the trans compartment (virtual ground). Chart as well as XY-recorder were used to record the membrane current. Voltages in the charts and diagrams refer always to the potential of the cis compartment versus trans compartment. Thus, a positive membrane current is defined as a flux of cations from the cis- into the trans compartment and/or a anion flux in the opposite direction.

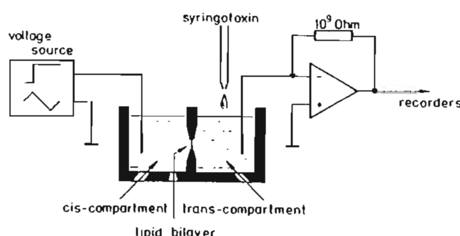

Fig. 1 : Schematic outlay of the experimental set-up

Aqueous solutions were prepared from p.a. grade chemicals and buffered by 10 mmol/1 TRIS-HCl, or HEPES-KOH, respectively, according desired pH.

The specific conductance of the solutions used was determined with a three electrode conductometer (RADELKIS, Hungary).

Syringotoxin was a gift of Prof. J.E. DE VAY (University of California in Davis). For the experiments a stock solution of 1.2 mg per 1 ml distilled water was used. Samples of this stock were added to the aqueous solution of the trans compartment to get final ST concentrations between 0.6 to 4.5 µg/ml.

RESULTS AND DISCUSSION

The electrical conductance of the unmodified bilayer is very low (approx. 10 to 20 pS). The presence of syringotoxin caused a significant increase of the membrane conductance which exponentially depended on its final concentration in the trans compartment, fig. 2. At concentrations below 1 µg/ml the membrane current changed in well defined entities, fig. 3, proving the incorporation of ion channels of identical size into the lipid membrane. This is taken as the primary mechanism of the increased membrane conductance induced by syringotoxin.

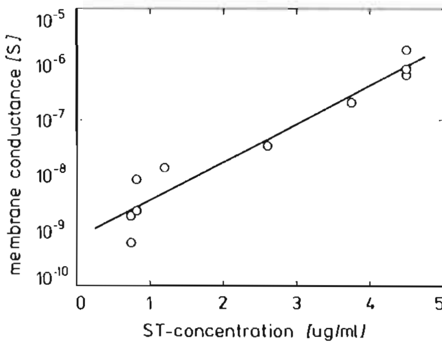

Fig. 2 :
Electrical conductance of the lipid bilayer in dependence on the ST concentration in the trans compartment.
Aqueous solution : 0.1 mol/1 KCl, pH 5.5

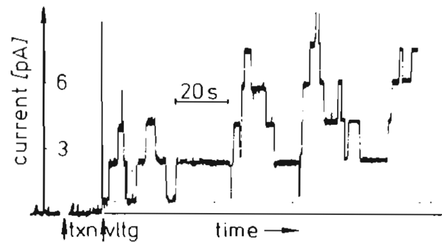

Fig. 3 :
Ion channels induced in the lipid bilayer upon adding ST to the aqueous solution of the trans compartment (0.75 µg/ml). First arrow marks the addition of the toxin sample, the second marks the application of a membrane voltage of +10 mV.
Aqueous solution : 1 mol/1 KCl, pH 5,5. The average single channel conductance is about 170 pS.

There was a linear relation between the single channel conductance and the specific conductance of the aqueous solutions used, fig. 4. On the other hand no obvious dependence of the channel conductance increment on the membrane forming lipid type was observed. Since single ST-channels possess linear IV-curves we can conclude that their transfer rate is not limited by possible ion-channel interactions but depends solely on the availability of ions at the channel entrance.

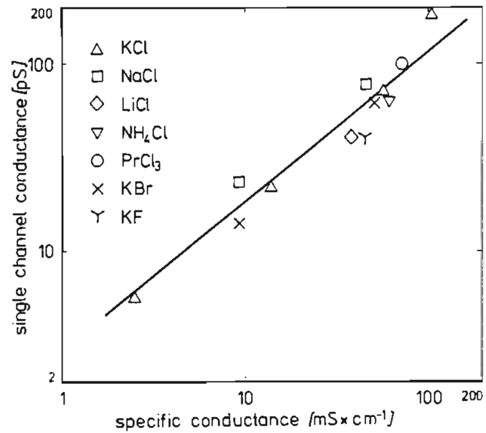

Fig. 4 : Relation between single channel conductance and specific conductance of the respective aqueous solution used.

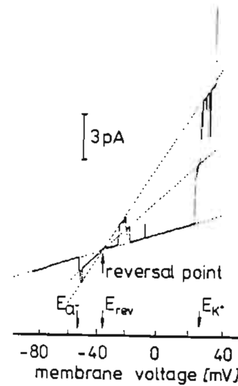

Fig. 5 : IV-curves related to different numbers of ion channels in the presence of a transmembrane activity gradient of 10:1. Channel currents reverse their direction at E_{rev} = 37.7 mV. E_{C1} and E_K denote the Nernst potentials for $C1^-$ and K^+.

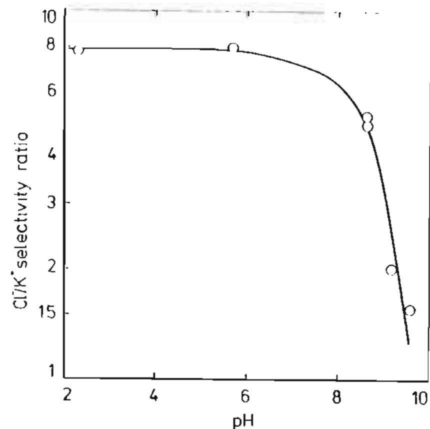

Fig. 6 : Dependence of the anion/cation selectivity ratio on the pH of aqueous solutions used. This diagram refers to experiments performed with KCl only.

In the case of a transmembrane salt gradient (higher concentration, 0.5 mol/1 KCl, in the trans compartment) individual IV-curves corresponding to different numbers of channels present in the bilayer are intersecting each other at a negative membrane voltage, fig. 5. The potential of this reversal point is for a given activity gradient determined by the anion-cation selectivity of the channel. From the relation between the reversal potential and the Nernst potentials of the respective ion species the selectivity ration can be estimated. As fig. 5 shows the channel displays a considerable anion selectivity the extent of which depends strongly on the valency of the cation used. While in the case of KCl and KBr the selectivity ratio is 8 and 7.5 respectively, it reaches already 11 for $MgCl_2$. In the case of $PrCl_3$ the reversal potential is identical with the Nernst potential for chloride which means that the channel is permeable solely for Cl^- -ions. A positive net-charge inside the channel or near its entrance is expected to prevent cations to enter the channel.

Analysing the amino acid composition of syringotoxin GROSS *et al.* (1977) have found that this toxin tentatively contains threonine, serine, glycine, ornithine and a further unidentified basic amino acid in an equimolar ratio. The secondary amino groups of ornithine and the unidentified acid are considered as the most likely source of the positive charge at lower pH values. With increasing pH the protonization of these groups should decline and with its diminishing charge the channel looses its ability to discern between anions and cations. At pH values above 6 the selectivity ration starts to decline and near pH 10 the channel becomes practically non selective.

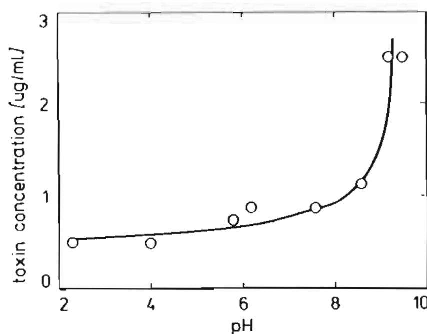

Fig. 7 : Dependence of the channel forming performance of ST on the pH in the aqueous solutions.
Since increasing pH values suppress the positive net-charge of syringotoxin its incorporation rate strongly declines and higher toxin concentrations are necessary to induce same conductance effects in the bilayer

Besides of the impact on the selectivity behaviour the charge plays an important role in the process of incorporation of ST-channels into the lipid bilayer. As fig. 7 shows the amount of toxin required to induce one or two ion channels in the lipid membrane strongly increases with the pH value of the aqueous solutions used. For instance the ST concentration which causes some channel events at a time at pH 9 would be sufficient to rise the membrane conductance by orders of magnitude at pH 5.5 (compare with Fig. 2).

Already SURICO & DE VAY (1982) have suggested that the toxic activity of syringotoxin on isolated maize mitochondria is due to its ability to depolarize affected membranes. The results of our experiments have brought strong evidence that such a depolarization might be easily caused by incorporation of a sufficient amount of ion channels into the membrane. Since the induction of ion channels does not depend on the lipid used and no ion specific effects have been observed there is good reason to expect that syringotoxin acts on the lipid moiety of biomembranes in a manner similar to that observed in our bilayer experiments.

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Pectic enzyme patterns in a bacterial and a fungal phytopathogen

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Abstract

Extracellular pectic enzymes produced by the phytopathogens *Fusarium oxysporum* f.sp. *ciceris*, *Pseudomonas marginalis* pv. *alfalfae* and pv. *pastinacae* were analyzed with IEF, their action pattern was studied by TLC. The fungus produces a very complex enzyme battery comprising pectic lyases, polygalacturonases and pectinesterases in many isoenzyme forms for the latter two types, while both bacterial strains produce one isoenzyme form of pectic lyase and pectinesterase. Both systems can however degrade polygalacturonic acid down to the monomer.

Keywords : cell wall degrading enzyme, *Fusarium oxysporum*, pectic enzyme, phytopathogen, *Pseudomonas marginalis*

Introduction

Pectic enzymes of phytopathogens play different roles in the pathogen's interactions with the host plant : they can facilitate entry into the plant by maceration of its tissue, they give access to pectic compounds as a carbon source in the host cell wall, they may also liberate biologically active plant cell wall polysaccharide fragments.

Pectic enzymes comprise different catalytic types. Pectic lyases (acting through β–elimination) and polygalacturonases (hydrolyzing the glycoside bond) are both classes of depolymerizing enzymes, while the pectinesterases demethylate

methyl–esterified carboxyl groups of the uronic moiety. Often one organism produces a battery of different pectic enzymes.

Fusarium oxysporum f.sp. *ciceris* (FOC) is a fungal pathogen colonizing the vascular tissue of chickpea, thus causing vascular wilt disease and eventually death of the host. *Pseudomonas marginalis* pv. *alfalfae* (PMA) and pv. *pastinacae* (PMP) are bacterial pathogens of alfalfa (root browning, stunting) resp. parsnip (firm rot in roots, soft rot in petioles).

Objective

The objective was to compare the composition of the pectic enzyme battery of a fungal pathogen vs. bacterial pathogens : occurrence of different catalytic types, isoenzyme profiles, action pattern.

Organisms and strains

Fusarium oxysporum Schlecht. emend. Snyd. & Hans. f.sp. *ciceris* (Padwick) Snyd. & Hans : fungal pathogen of *Cicer arietinum* L. (chickpea) : race 1, kindly provided by Dr M.V. Reddy (ICRISAT, India).

Pseudomonas marginalis pv. *alfalfae*, bacterial pathogen of *Medicago sativa* L. (alfalfa) : strain LMG 2214 and *P. marginalis* pv. *pastinacae*, bacterial pathogen of *Pastinaca sativa* L. (parsnip) : strain LMG 2238, both kindly provided by Dr P. De Vos (LMG, Gent).

Materials and methods

– ***Fusarium oxysporum* f.sp. *ciceris*, race 1** was grown in liquid modified Armstrong medium (BOOTH, 1971) without $Ca(NO_3)_2$, adjusted at pH 6.5 before and after autoclaving. NH_4NO_3 was added at 10 g/l. Pectin (Sigma) was added at 0.5 % as the carbon source. Media were inoculated with a 0.4 cm^2 agar plug of the margin of an actively growing fungal culture (Potato dextrose agar, GAMS *et al.*, 1987). Culture supernatants were collected for enzyme assay at 7 days by suction filtration over filter paper, then centrifuged at 2000 g.

– ***Pseudomonas* strains** were grown in succinate medium (g/l K_2HPO_4 6, KH_2PO_4 3, $(NH_4)_2SO_4$ 1, $MgSO_4.7H_2O$ 0.2, succinic acid 4, pH 7.0) or in the same medium with substitution of succinate with 6.5 g/l pectin. Inoculation was from a pre–culture in the pectin–substituted succinate medium. Culture supernatants were collected for enzyme assay at 7 days by centrifugation at 2000 g.

– **Screening for depolymerizing pectic enzyme activity** was done with crude culture supernatants of about 60 bacterial strains directly applied on the

overlay sheets as described below for IEF (depolymerizing activity) or on an agarose gel in α–naphtyl acetate and Fast Blue RR, as described below for IEF (esterase).

– Culture filtrates were **dialyzed** against twice deionised water at 4°C (1:50, repeated) and **lyophilyzed**. All storage was at –20°C.

– For more concentrated samples **affinity chromatography** was performed on an epichlorohydrin–cross linked alginate (ECH/AGU=2.35) column (1.5 x 24 cm). Dialyzed, lyophilyzed samples were redissolved in water and loaded onto the column at pH 4.2 sodium acetate buffer 0.1 M. Pectic enzymes were eluted in a 0.1 M sodium acetate buffer pH 6.0 with 0.5 M NaCl.

– **Horizontal thin layer IEF** was performed with electrofocusing gels pH 3.5–9.5 (Ampholine, LKB–Pharmacia), 10°C, 10 W for about two hours, with a set of pI standards (pI 4.65–9.6 from BioRad). **Depolymerizing enzyme activity after IEF** was detected by an overlay technique (adapted from BERTHEAU *et al.*, 1984) with a 1 % agarose gel overlay on an agarose GelBond film (LKB–Pharmacia) containing enzyme substrate (polygalacturonic acid 0.1 %, 50 mM potassium acetate buffer pH 4.5 with 10 mM EDTA for hydrolases or 50 mM Tris–Cl buffer pH 8.5 for lyases) after a buffer wash (2x500 ml, 2x15') at the appropriate pH. Overlay time was two hours. The overlay was developed by Ruthenium Red (0.03 % in water). Enzyme activity is shown by the clearing of substrate which is stained red during development. **Esterase activity** was detected in the IEF gel itself by the method of SZECSI & HORNOK (1986) after two washes in the appropriate buffer system. The method is not specific for ***pectin***esterase, but detects other esterases as well.

– **Polygalacturonase activity** was assayed in the reaction mixture of COLLMER *et al.* (1988) and reducing sugars were measured with GROSS' method (1982) using 2–cyanoacetamide. **Pectic lyase activity** was assayed by the increase of absorbance at 232 nm in Tris–HCl 50 mM pH 8.5 with polygalacturonic acid 0.2 % at 30°C. **Pectinesterase** activity was assayed by the pH–decrease method of FÖRSTER (1988).

– **Thin layer chromatography (TLC)** was performed on microcrystalline cellulose plates (Merck HPTLC 0.1 mm) with ethyl acetate:n–butanol:formic acid:water=1:3:5:2 solvent system (method MARKOVIC & SLEZARIK, 1984). Each sheet was run twice. Standards were mono–, di– and trigalacturonic acid (Sigma). The sheets were developed in saturated lead acetate spray and with subsequent baking for 20' at 80°C. Samples were overnight incubation mixtures at 30°C in 50 mM sodium acetate buffer (pH 5 or 8.5) containing 0.5 % orange polygalacturonic acid (Sigma).

I : polygalacturonase

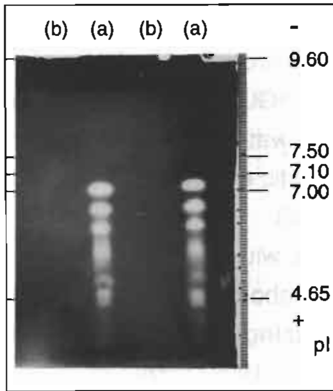


Figure : Pectic enzyme profiles after iso-electric focusing (with affinity chromatography concentration step for FOC) : Ampholine pH 3.5-9.5.

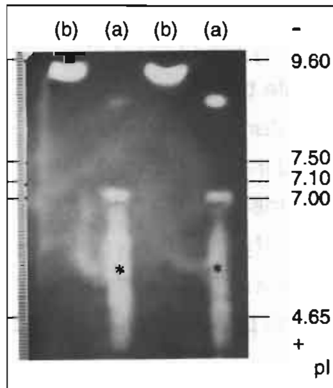
organisms : (a) FOC (b) PMA.

+ = anode; − = cathode.

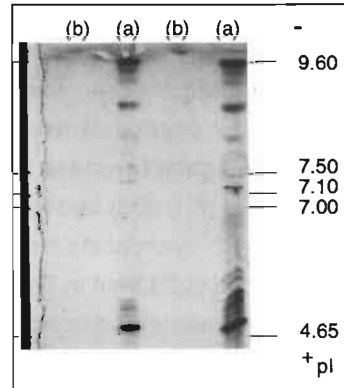
pI standards :
phycocyanine (pI 4.65);
equine myoglobin (pI 7.00);
human hemoglobin A (pI 7.10);
human hemoglobin C (pI 7.50);
cytochrome c (pI 9.60).

* = residual polygalacturonase activity at pH 8.5.

II : pectic lyase



III : pectinesterase



Results

Pectic enzyme activity is undetectable in FOC cultures with glucose as a C-source, (pectin)esterase is undetectable in PM cultures with succinate substituted for pectin while pectic lyase is still produced (results not shown). If grown on pectin as a sole carbon source, both the fungi and the bacteria produce pectic enzymes, which are found in the culture supernatant. The pectic enzyme pattern is entirely different in *Fusarium* and *Pseudomonas marginalis* pathovars, which were the only bacteria

in a series of about 60 strains screened (mainly pseudomonads, results not shown), shown to produce depolymerizing pectic enzymes in considerable amounts.

Whereas FOC produces a wide range of acid to neutral isoenzymes of polygalacturonase (as indicated by IEF, figure) and one single alkaline form of pectic lyase, PM shows no or traces of polygalacturonase and one single alkaline form of pectic lyase. Esterase is produced in a wide range of alkaline to acid isoenzyme forms by FOC, but only weakly in one highly alkaline form in PM supernatants. Patterns for both PM pathovars were not distinguishable. Since the esterases all bind along with other pectic enzymes on cross-linked alginate and since they are not detectable if glucose (FOC) or succinate (PM) is given as the C-source instead of pectin, we assume they are all pectinesterases. All enzyme activities detected after IEF were confirmed in the quantitative enzyme assays, except for the very low pectinesterase activity in PM. As far as the comparison is valid, the absolute levels of pectic lyases under these culture conditions are considerably higher in PM than in FOC supernatants.

If the entire pectic enzyme battery is used to extensively degrade polygalacturonic acid, the polymer can be degraded up to the monomer for both the fungus and the bacteria.

Conclusions

Both pathovars of *Pseudomonas* produce one major depolymerizing pectic enzyme in large amounts and apparently one (pectin)esterase, as opposed to *Fusarium* with a complex mixture of different enzymes, comprising different catalytic types (pectic lyases, polygalacturonases, pectinesterases), often in many isoenzyme forms. The role of this cell wall degrading strategy in the interaction of the phytopathogen with its host remains to be explained. TSUYUMU *et al.* (1991) reported for soft rot *Erwinia* that two lyases active on high respectively low methyl-esterified pectic compounds act synergistically and are both needed to fully degrade the polymer. Here, the enzyme battery of PM is sufficient for full degradation of polygalacturonic acid, *in planta* pectinesterase might also pave the way for pectate lyase on methyl-esterified stretches. It is difficult to conceive that the considerable complexity of the FOC system is necessary solely for the release of galacturonic acid monomers, when this task is performed as effectively with the simple PM system. The difference may therefore reflect different strategies of both types of pathogens : colonization of the complex vascular tissue by FOC and more superficial infection and maceration by PM. The roles of the single enzymes must still be elucidated. KEON *et al.* (1987) suggest that high pectic enzyme pI may allow binding to the acidic substrate at physiological pH. This can however not explain the very high pI of pectinesterases, for which polygalacturonic acid or acidic polymer

stretches are not the substrate. Yet, care should be observed in extrapolating the results to *in vivo* conditions : surface and xylem conditions *in planta* may be different from liquid cultures, e.g. because of the substrate complexity and the host's response.

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Reduced activity of *Erwinia chrysanthemi* pectinases after inoculation of potato plants resistant to soft rot

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ABSTRACT

Erwinia chrysanthemi (Ech) causes different kind of disease symptoms on potato plants: maceration of the potato tuber tissue, stem rot or blackleg. Ech produces several extracellular enzymes that can degrade components of the plant cell wall: pectinases, cellulases and proteases. Pectate lyases (PL) seem to play a key function in the pathogenicity.

In order to study the mechanism of Ech pathogenicity, tubers and *"in vitro"* plants of somatic hybrids of *Solanum tuberosum* and *Solanum brevidens* (resistant to soft rot) and commercial cultivar Katahdin (susceptible to soft rot), were inoculated with the bacteria. The disease severity, bacterial multiplication and PL activity in rotting tissue were determined during six days after inoculation. Rotting was significantly lower in tubers and plants of somatic hybrid. However, the number of bacteria was similar in both resistant and susceptible tissues. On the other hand, the PL activity showed significantly lower level in resistant tubers. Therefore, it seems that Ech could grow easily in the tissue of resistant or susceptible lines, but the PL production or activity was reduced in resistant tubers. The changes of PL activity in the *"in vitro"* plants are discussed.

Key words: *Erwinia chrysanthemi*, soft rot, somatic hybrids, potato .

INTRODUCTION

Soft rot of potato tubers and stems caused by the bacteria from the genus *Erwinia* often results in losses in the growing potato crop as well as after harvest in decay of tubers in storage and in transit. *Erwinia chrysanthemi* produces several extracellular enzymes that can degrade plant cell walls. Among them, pectinolytic enzymes were shown to be the most important in pathogenicity (BASHAN &

BATEMAN, 1975, PEROMBELON & KELMAN, 1987). Pectin is first demethylated by the pectin methylesterase (PME) and then polygalacturonic acid is hydrolyzed by PL. Ech produces one PME and five isoenzymes of PL. The genes coding for pectin degrading enzymes are organized in two clusters on the Ech chromosome. Each gene constitutes an independent transcriptional unit (REVERCHON *et al.*, 1986, HUGOUVIEUX-COTTE-PATTAT & ROBERT-BAUDOUY, 1989, RIED & COLLMER, 1986).

Disease severity could be limited by several factors such as: inhibition of bacterial growth and multiplication, inhibition of the biosynthesis of pectinolytic enzymes or inactivation of the bacterial enzymes by factors originating from plant tissue. Tubers and *"in vitro"* plants of different varieties, characterized as resistant or susceptible to Ech, were used for the study of the pathogenicity mechanism. Our study was concentrated on some of the factors that could limit disease symptoms: bacterial multiplication and pectinase activity in the rotting tissue.

MATERIALS AND METHODS

Plant material

Tubers of potato cultivar Katahdin were obtained from cultivar evaluation plots established in the Institute for Potato Research, Bonin, Poland. The clonal lines of hexaploid somatic hybrid used in this study were produced by the protoplast fusion of diploid *Solanum brevidens* cells with those of tetraploid *Solanum tuberosum*. This material was obtained from Dr. J. Helgeson, University of Wisconsin-Madison. The production and field evaluation of somatic hybrids has been described previously (AUSTIN *et al.*, 1986). Plants of somatic hybrid, were grown in the same experimental trial as cultivar Katahdin. Tubers of somatic hybrids were previously shown to be resistant to bacterial soft rot (AUSTIN *et al.*, 1988, LOJKOWSKA & KELMAN, 1990).

Bacterial culture

The derivative of the *Erwinia chrysanthemi* strain 3937, used in this study was the mutant A988, resistant to kanamycin, obtained in Laboratoire de Génétique Moléculaire des Microorganismes, INSA, Lyon. Suspensions for inoculations were prepared from cultures following established procedures (CUPPELS & KELMAN, 1974).

Screening for soft rot resistance

Test on tubers

Tubers (free of any obvious mechanical damage or disease) were washed and surface sterilized. Sterile polypropylene pipette tips containing 50 μl of bacterial suspension (5 x 10^7 cfu/ml) were pressed in a randomized manner into the upper surface of each tuber to the depth of 10 mm (MAHER & KELMAN, 1983). In control samples, sterile water was injected to the tubers. Tubers from each variety were incubated at 30°C in a chamber at 100% relative humidity. Determination of the disease severity, number of bacteria and PL activity were performed after 3 days of incubation or every 24 hours during 6 days of incubation. Tubers were sliced vertically through the inoculation point and the width of decayed tissue was measured. Rotting index is described as a mean width of decayed tissue. For determination of bacterial survival, samples of tissue from 10 plants or tubers of each accession were ground with mortar and pestle in a sterile phosphate buffer. Dilution plating was performed in duplicate on CPG + kanamycin agar plates (50 μg/ml of medium). Rotting tissue was collected and lyophilized for later determination of PL activity.

Test on *"in vitro"* plants

"In vitro" plants were grown in a phytotron about four weeks. A petiole and leaf base on each plant (third from tip) were crushed with tweezers. Then, the crushed surface was covered with 50 μl of suspension of Ech (approx 10^8 cfu/ml). Disease severity was evaluated by the following system: no disease symptoms = 0; single leaf wilting = 1; several leaves wilting = 2; wilting of whole plant = 3; wilting and stem rot, but seedling survives inoculation = 4; stem rot and death of seedling = 5. The disease index was calculated as a mean value for all inoculated seedlings from each line. Determination of the disease severity, number of bacteria and PL activity were performed after 5 days of incubation or every 24 hours during 8 days of incubation. Survival of bacteria and PL activity were determined in the same manner as for tubers.

Determination of PL activity

Pectate lyase activity was assayed in the extracts of the lyophilized tuber or *"in vitro"* plant tissue. PL activity was determined by following the degradation of polygalacturonic acid to unsaturated products that absorb at 235 nm (MORAN & STARR, 1968). Unit of PL activity is expressed as μmol of unsaturated products liberated per min, either per g of fresh weight (FW) or per 10^9 cfu of bacteria.

RESULTS AND DISCUSSION

Tissues of tubers and *"in vitro"* plants of somatic hybrids were significantly more resistant to *Erwinia* infection than tissues of cultivar Katahdin (Fig.1A). A tendency for a better growth of bacteria was observed but the differences between the numbers of bacteria invading the "resistant" or "susceptible" tissues were no significant (Fig.1B) In case of the tuber tissue, no significant difference in PL activity per gram of the tissue was observed. In contrast, PL activity was significantly higher in *"in vitro"* plants of Katahdin than in *"in vitro"* plants of somatic hybrid (Fig.1C). Similar results were obtained for the PL activity expressed per 10^9 cfu of Ech.

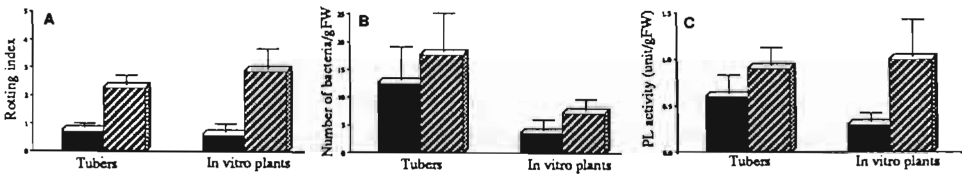

Fig. 1. Comparison of the infection process in soft rot resistant (■) and susceptible (▨) tissues. A. Disease severity; tubers were infected with 50 µl of bacterial suspension (5 x 10^7cfu/ml) and incubated in a dew chamber at 30°C. Rotting index is the mean width of decayed tissue divided by 5. *"In vitro"* plants were inoculated with 50 µl of bacterial suspension (10^8 cfu/ml). For rotting index we used a scale from 0 to 5, where 5 means dead plant. **B.** Number of bacteria; bacteria were isolated from inoculated tissue. For tubers the shown values should be multiply by 10^9 cfu but for *"in vitro"* plants by 10^8 cfu. **C.** PL activity was assayed in extracts of the lyophilized tissues. PL activity is expressed in units per g of FW.

In order to observe the kinetic of the changes in the inoculated tuber tissues, we followed the infection process during six days. Disease severity was always about twice lower in the tubers of the somatic hybrid than in the tubers of the cultivar Katahdin (Fig.2). As it was expected, the number of bacteria isolated from both tested tissues was similar. During the first 3 days

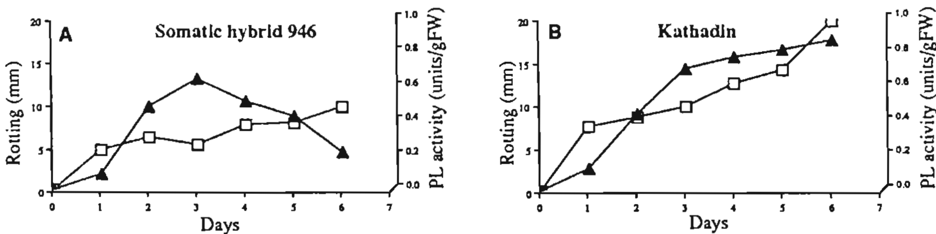

Fig. 2. Dynamic of the changes in disease severity and PL activity after inoculation of tubers. Rotting index (□); PL activity (▲). All comments as on Fig. 1.

after inoculation, PL activity increased similarly in both resistant and susceptible tubers (Fig.2). Then PL activity still gradually increased in susceptible tubers to reach 0.8 units/gFW (Fig.2B). In opposite, in resistant tubers PL activity dropped down to 0.2 units/g FW after six days i.e. to the same level as at the first day (Fig.2A). These results indicate that the tissue of the resistant tubers could contain factor(s) inhibiting the activity of PL enzymes. It was shown earlier that the somatic hybrid tissue has a high activity of polyphenol oxidase and peroxidase (LOJKOWSKA & HOLUBOWSKA, 1992). These enzymes could play a role in the inactivation of PL (COLLINGE & SLUSARENKO, 1987, LYON, 1989).

To follow the dynamic of the changes after inoculation of *"in vitro"* plants, the disease severity was determined during eight days. The disease progressed rapidly in the Katahdin plants during the first four days (Fig.3B). During the same time, we observed a very low level of rotting in the somatic hybrid plants (Fig.3A). However, after 4 days, the level of rotting increased quickly also in somatic hybrid plants. It seems that *Erwinia* can break the defense system of those plants few days after inoculation.

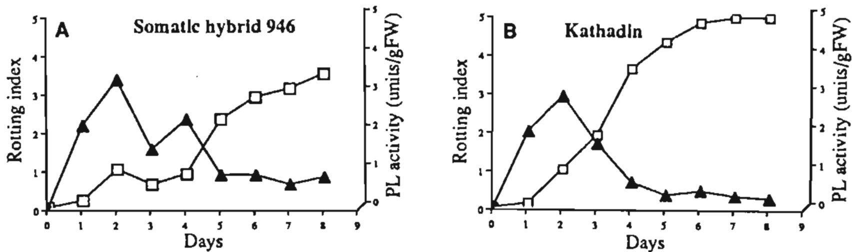

Fig. 3. Dynamic of the changes in disease severity and PL activity after inoculation of *"in vitro"* plants. Rotting index (□); PL activity (▲). All comments as Fig.1.

The change of the PL activity was similar in resistant and susceptible plants: the enzyme activity sharply increased during the first two days after inoculation, then the PL activity decreased in both cases (Fig.3). Before inoculation bacteria were grown on a minimal medium in non inducible conditions. The increase of the PL activity was observed in both resistant and susceptible tissue. The transcription of pectate lyase encoding genes is inducible by pectin derivative compounds (HUGOUVIEUX-COTTE-PATTAT *et al.*, 1986). Therefore, PL induction occurs as a result of pectin degradation, in both resistant and susceptible plants.

In case of susceptible plants, we observed a decrease of the PL activity when the rotting was still progressing. These results could be explained by a delay between enzyme action and perceptible rotting or by a disappearance of

enzyme activity as a results of shortage of substrate (all tissue is macerated). In the resistant plants disease symptoms are weaker, even with high PL activity.

Our results suggest that resistance of the somatic hybrids tissue to soft rot is not the effect of a repression of the genes coding for pectate lyases. We propose two hypothesis explaining why the disease severity is weaker in the tissue of somatic hybrids tubers and *"in vitro"* plants. The first is that resistant plant tissues possess factor(s) that could inhibit PL activity by inactivating the enzyme produced by *Erwinia* (MAHER & KELMAN, 1983). The other possibility is that pectin of resistant plants is highly methylated (PEROMBELON, unpublished data) and as such could not be degraded by pectate lyase. In this case the activity of the pectin methylesterase could be the limiting factor.

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Xanthine oxidase (XOD) activity in bean leaves infected by *Pseudomonas syringae* pv. *phaseolicola*

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Abstract

Xanthine oxidoreductase exhibiting a superoxide-producing activity (xanthine oxidase, E.C. 1.2.3.2) was evaluated in the first trifoliate leaves of *Phaseolus vulgaris* L. (cv. Red Mexican 34) during infection with race 1 (avirulent) and race 2 (virulent) of *Pseudomonas syringae* pv. *phaseolicola* inoculated at 10^8 cells/ml. Enzyme activity was assayed in crude extracts of leaves collected 5, 12, 24 and 48 hours after inoculation. The activity of xanthine oxidase was unchanged in both incompatible and compatible interactions at 5 and 12 h after inoculation. Only the avirulent race 1 induced a significant increase in xanthine oxidase at 24 and 48 h after inoculation (130% and 150% of the control, respectively), when cell collapse and necrotic symptoms were evident. Allopurinol, a potent and specific inhibitor of XOD, when soil applied (by irrigation) 8 days before inoculation at 400 µM to bean plants, did not inhibit hypersensitive expression. These findings indicate that purine oxidative catabolism is not involved in the pathogenic process leading to hypersensitive response in the *Pseudomonas syringae* pv. *phaseolicola* - *Phaseolus vulgaris* complex.

Key words: *Pseudomonas syringae* pv. *phaseolicola*, xanthine oxidase, allopurinol, hypersensitive reaction.

Introduction

Xanthine oxidoreductase (XOD) is an aerobic purine degrading enzyme which catalyzes the oxidation of hypoxanthine to xanthine and xanthine to uric acid (for pertinent literature see MONTALBINI, 1992a). Two xanthine-oxidizing enzymes exist depending on whether the substrate is oxidized by O_2 (xanthine oxidase,

E.C. 1.2.3.2, type O enzyme) or by NAD^+, ferredoxin or methyl viologen (xanthine dehydrogenase, E.C. 1.2.1.37, type D enzyme).

Little research has been conducted on the response of xanthine-oxidizing enzymes during infection by pathogenic organisms. Recently a superoxide-producing xanthine oxidase from bean leaves has been shown to be involved in the incompatible interaction between *Phaseolus vulgaris* and *Uromyces phaseoli* (MONTALBINI, 1992 a, b).

We recently reported that *Pseudomonas syringae* pv. *syringae* infection on bean leaves increased xanthine oxidase activity slightly only during the compatible interaction (MONTALBINI & BUONAURIO, 1991).

The aim of the present study was to investigate the role of xanthine oxidase in both incompatible and compatible combinations between *Phaseolus vulgaris* and *Pseudomonas syringae* pv. *phaseolicola*.

Materials and methods

Phaseolus vulgaris L., cv. Red Mexican 34 and *Pseudomonas syringae* pv. *phaseolicola* strain GSPB 1552 (race 1) and strain GSPB 1495 (race 2), kindly provided by Dr. K. Rudolph (Institut für Pflanzenpathologie und Pflanzenschutz, Göttingen University, Germany), were used for the experiments.

Plant growth and bacterial inoculation procedures were as previously described (BUONAURIO & MONTALBINI, 1991).

Allopurinol [4-hydropyrazolo (3,4-d) pyrimidine] treatment was started when primary bean leaves were well developed and the first trifoliate leaves started to appear. A 400 μM solution was applied daily as a soil drench. Eight days after the treatment, the first trifoliate leaves were inoculated. The effect of allopurinol on bacterial growth *in vitro* was also tested. For this purpose, allopurinol was included in nutrient broth at 400 and 800 μM concentrations and bacterial growth, measured as optical density at 660 nm, and compared with a control where only nutrient broth was used.

Electrolyte leakage was determined as already described (BUONAURIO & MONTALBINI, 1991).

Bacterial multiplication *in planta* was quantified by the number of colonies obtained by plating several dilutions of leaf homogenate on nutrient agar (RHODES, 1959).

Xanthine oxidase activity was assayed in a crude bean leaf extract after polyacrylamide gel electrophoresis according to the method of MENDEL & MÜLLER (1976), as previously described (MONTALBINI & BUONAURIO, 1991).

Protein was determined with the Folin-phenol reagent (LAYNE, 1957) using bovine serum albumin as standard.

Results

Time-course of symptom expression

Infection with avirulent race 1 was characterized by macroscopic cell collapse, which appeared 10-12 h postinoculation and reached its highest intensity at 24 h. The collapsed areas became necrotic at 24 to 48 h. Virulent race 2 infection was distinguished by numerous water-soaked lesions which were first noted 48 h after inoculation.

Effect of P. syringae *pv.* phaseolicola *infection on XOD activity*

Xanthine oxidase activity was unchanged in both the incompatible and compatible interactions at 5 and 12 h after inoculation. Only avirulent race 1 induced a significant increase in xanthine oxidase at 24 and 48 h postinoculation (130% and 150% of the control, respectively), when cell collapse and necrotic symptoms were evident (Fig. 1)

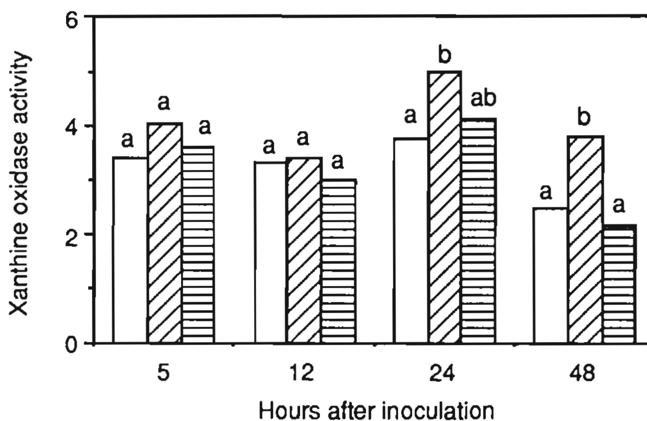

Figure 1. Time-course of xanthine oxidase activity in bean leaves infected with *Pseudomonas syringae* pv. *phaseolicola*.
Each value is the mean of 3 independent experiments. Columns within each hour, capped with the same letters are not significantly different (P=0.05) according to Duncan's multiple range test.

☐ Healthy; ▨ infected with avirulent race 1; ▤ infected with virulent race 2.

Effect of allopurinol on symptom expression, electrolyte leakage and bacterial multiplication.

Allopurinol treatment did not abolish the hypersensitive response induced by infection with the avirulent race of *P. syringae* pv. *phaseolicola*, but provoked a more rapid and intense appearance of necrotic symptoms, which were fully expressed from 24 h postinoculation.

Untreated infected leaves manifested an increase in electrolyte leakage only in the incompatible interaction at the beginning of collapse (153 % of the healthy control at 10 h after inoculation) and at the onset of necrosis (223% of the healthy control at 24 h after inoculation) that was no longer detectable 48 after inoculation, when the necrotic process reached its maximum expression (Fig. 2). In the incompatible

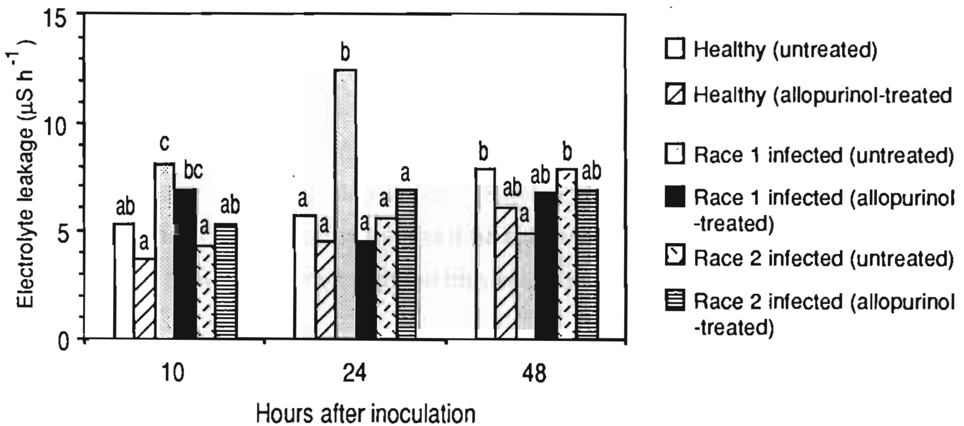

Figure 2. Effect of *Pseudomonas syringae* pv. *phaseolicola* infection on electrolyte leakage in untreated and allopurinol-treated bean leaves.
For further details see legend of Fig. 1

interaction, in allopurinol-treated infected leaves, a significant increase in electrolyte leakage (183% of the control) was only observed 10 h postinoculation (tissue collapse) if data were compared to the correspondent healthy-allopurinol-treated plants. In contrast to untreated race 1- infected leaves (incompatible interaction), treated leaves infected with the same race did not show any changes in electrolyte leakage 24 h postinoculation (Fig. 2), probably because, as previously stated, the necrotic process was already fully expressed in the allopurinol-treated leaves at this time.

The bacterial multiplication rate measured at 0, 24 and 48 h after inoculation was lower for the avirulent than the virulent race (Fig 3).

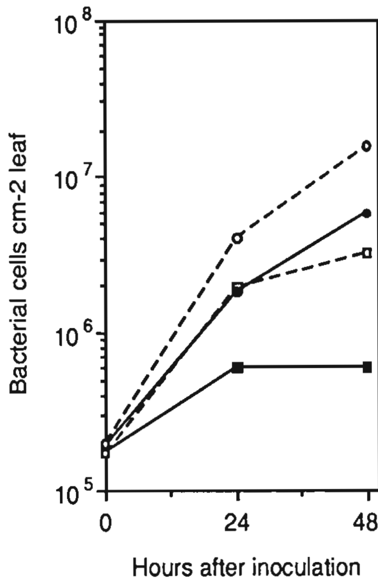

Figure 3. Multiplication of *Pseudomonas syringae* pv. *phaseolicola* in untreated and allopurinol-treated bean plants.

--□-- Untreated plants inoculated with avirulent race 1

—■— Allopurinol-treated plants inoculated with avirulent race 1

--○-- Untreated plants inoculated with virulent race 2

—●— Allopurinol-treated plants inoculated with virulent race 2

Although allopurinol did not inhibit *P. syringae* pv. *phaseolicola* growth *in vitro*, in allopurinol-treated leaves, there was a decrease in bacterial growth of both the virulent and avirulent races. However, the decrease was much more pronounced in avirulent race, which displayed no further growth from 24 to 48 h after inoculation, probably, as previously stated in the section realtive to the effect of allopurinol on symptom expression, in relation to earlier and more marked cell necrosis.

Discussion

During the hypersensitive response induced in bean leaves by the avirulent race 1 of *P. syringae* pv. *phaseolicola*, an increase in xanthine oxidase activity was detected only late after infection, when necrotic symptoms were already established. When allopurinol, a specific and potent *in vitro* and *in vivo* inhibitor of xanthine oxidase (FUJIHARA & YAMAGUCHI, 1978; HILLE & MASSEY, 1981), was applied to bean plants by root absorption, it failed to prevent necrotic process associated with hypersensitive reaction. Probably due to the bacterial growth inhibition in race1-infected allopurinol-treated plants (Fig.3), electrolyte leakage enhancement was less intense and lasted less than in race 1-infected untreated plants. However, it seems that xanthine oxidase is little or not implicated in the hypersensitive reaction induced by *P. syringae* pv. *phaseolicola* in bean plants. These findings contrast with those obtained in the incompatible interaction between *Phaseolus vulgaris* and *Uromyces*

phaseoli (MONTALBINI, 1992a), where the effectiveness of allopurinol in abolishing hypersensitivity expression (MONTALBINI, 1992b) led us to propose a cause-effect relationship between the increase in superoxide-producing xanthine oxidase activity and hypersensitivity cell death.

The increase in lipoxygenase-linked lipid peroxidation observed in the incompatible interaction between *Phaseolus vulgaris* and *P. syringae* pv. *phaseolicola* before the appearance of symptoms (CROFT *et al.*, 1990) is more likely to be involved in determining the necrotic process associated with the hypersensitive response.

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Production of cutin-degrading enzymes by plant pathogenic bacteria

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ABSTRACT

A collection of two hundred and sixty-six filamentous (*Streptomyces*) and non-filamentous bacterial strains were screened for ability to produce enzymes (cutinases) which degrade the plant biopolyester cutin. The strains represented both plant pathogenic as well as saprophytic bacteria. Bacteria were grown in various liquid media supplemented with apple cutin. Extracellular esterase activity was determined by a spectrophotometric assay utilizing the model substrate p-nitro-phenyl butryrate. Culture fluids high in cutin-inducible esterase activity were examined for ability to release monomers from cutin. The released monomers were quantified by use of a novel HPLC technique developed in our laboratory. The identity of the major monomers released was confirmed by GC/MS analyses. By these techniques the culture fluids from our strains of *Streptomyces* spp. and three strains of *Pseudomonas aeruginosa* were demonstrated to contain cutinase activity. Addition of the nonionic detergent n-octyl glucoside to cutinase assay mixtures stimulated enzyme activity for culture filtrates of two of the seven strains. For *P. aeruginosa*, the highest levels of cutinase activity were observed at incubation temperatures below that optimal for growth.

KEYWORDS

Cutin, cutinase, esterase, *Streptomyces*, *Pseudomonas*.

INTRODUCTION

Most aerial parts of plants are covered with a protective barrier called the cuticle. The structural component of the cuticle is called cutin. Cutin is an insoluble biopolymer composed primarily of C_{16} and C_{18} hydroxy and epoxy fatty acids (KOLATTUKUDY, 1985). The cutin is embedded in and covered by a coating of cuticular waxes. The cuticle is thought to function as a protective barrier against desiccation as well as insect and microbial attack and is the major barrier for absorption of foliar applied agrichemicals.

Several fungal pathogens of plants have been shown to produce enzymes called cutinases which are capable of hydrolyzing cutin and a role for these enzymes in penetration of intact plant surfaces has been demonstrated. Several of these cutinases have been purified and characterized (KOLATTUKUDY, 1985). In contrast to the extensive literature on fungal cutinases, very little is known about bacterial cutinases. Only three bacteria (*Streptomyces scabies*, *Pseudomonas syringae* pv. *tomato* and *P. putida*) have been reported to exhibit this enzyme activity (BASHAN *et al.*, 1985; LIN & KOLATTUKUDY, 1980; SEBASTIAN , *et al.*, 1987).

Utilizing a novel HPLC technique developed in our laboratory for separation, identification and quantitation of released cutin monomers, we screened over 270 bacterial strains for ability to hydrolyze cutin.

MATERIALS AND METHODS

The bacteria tested and the number of strains of each were :
Bacillus brevis (5), *B. cereus* (3), *B. pumilus* (2), *B. sphaericus* (5), *B. subtilis* (4), *Clavibacter michiganensis* subsp. *michiganensis* (2), *Clavibacter michiganensis* subsp. *sepedonicus* (5), *Cytophaga johnsonae* (8), *Erwinia amylovora* (3), *E. ananas* (3), *E. carotovora* subsp. *atroseptica* (1), *E. chrysanthemi* (2), *E. cypripedii* (3), *E. herbicola* (10), *E. mallitovora* (1), *E. nigrifluens* (2), *E. rhapontici* (3), *E. rubrifaciens* (1), *E. stewartii* (1), *E. tracheiphila* (2), *E. uredovora* (3), *Pseudomonas aeruginosa* (10), *P. cepacia* (7), *P. chlororaphis* (1), *P. cichorii* (1), *P. fluorescens* (21), *P. marginalis* (7), *P. putida* (10,11), *P. syringae* pathovars *glycinea* (7), *lachrymans* (1), *morsprunorum* (1), *papulans* (5), *phaseolicola* (7), *pisi* (2), *syringae* (18), *tabaci* (1), *tagetis* (1), *tomato* (12), *P. viridiflava* (23), *Pseudomonas* spp. (2), *Streptomyces acidiscabies* (1), *S. badius* (2), *S. scabies* (1),

S. "scabies" (31), *S. ipomoea* (8), *S. viridisporus* (2), *Xanthomonas campestris* pathovars *campestris* (3), *cucurbitae* (1), *glycines* (3), *malvacearum* (2), *pelargonii* (1), *pruni* (2), *raphani* (1), *vesicatoria* (1) and *vignicola* (1).

Bacteria were first screened for production of esterase in various broth media containing 0.4% (w/v) of cutin prepared from mature fruits of apple cv. Golden Delicious or tomato. Cultures were incubated with shaking at room temperature and samples of culture fluids were periodically removed and tested for esterase activity by use of a spectrophotometric assay utilizing p-nitrophenyl butyrate as substrate (SEBASTIAN , *et al.*, 1987). Almost all known cutinases have the ability to hydrolyze this model substrate.

Assays for cutinase activity were done by addition of 0.5 ml culture fluid to 30 mg of apple cutin in 0.5 ml of 0.1 M potassium phosphate buffer, pH 8.0. The nonionic detergent n-octylglucoside was added to some assay mixtures (final concentration of 35 mM). Tubes were incubated with shaking in a water bath held at 27 C for 18 h and the released cutin monomers were extracted with chloroform- methanol (BLIGH & DYER, 1959). The cutin monomers were separated, identified and quantified by HPLC (GERARD, *et al.*, 1992). The identities of the major cutin monomers released were confirmed by GC/MS analysis.

RESULTS

Of the filamentous bacteria tested, culture fluids from four strains were found to exhibit both esterase activity as well as cutinase activity when the bacteria were grown in the presence of cutin (Table 1).

Table 1. Esterase and cutinase activity of *Streptomyces* culture fluids.

Bacterium	Strain	Medium[a]	Esterase (nmoles/min/ml)	Released monomers (mg/18h/ml culture fluid)
S. acidiscabies	ATCC 49003	TYE	220	1.38
S. "scabies"	ATCC 15485	GA	500	0.72
	IMRU 3018	TYE	850	2.30
S. badius	ATCC 19888	TYE	160	2.30

[a]TYE = tryptone-yeast extract broth, GA = glycerol-asparagine broth; plus cutin.

Production of esterase/cutinase by the four strains of *Streptomyces* was induced up to 17-fold by addition of cutin to the medium. Addition of the nonionic detergent n-octyl glucoside to the cutinase assay mixtures did not stimulate enzyme activity.

Of the 232 non-filamentous bacteria tested, only the ten strains of *P. aeruginosa* exhibited esterase activities over 500 nmoles/min/ml in broth medium supplemented with cutin and only three of the ten strains demonstrated significant (greater than ten-fold) induction of esterase activity by addition of cutin (Table 2).

Table 2. Maximum esterase activity of *Pseudomonas aeruginosa* during growth in NBY broth with or without apple cutin.

	Maximum esterase activity (nmoles/min/ml)	
Strain	- cutin	+ cutin
813	1200	866
1499A	577	7200
K799	125	56875
AK1012	1200	1100
PAO1.AK.957	350	1300
DAR 41352	780	26650
DAR 41353	980	900
DAR 41354	1000	780
DAR 41355	640	530
DAR 41360	1200	880

To determine if the three strains of *P. aeruginosa* which were high in cutin-inducible esterase production were also capable of cutin degradation, culture fluids were tested for their ability to release cutin monomers from apple cutin. HPLC and GC/MS analyses of assay mixtures confirmed the release of cutin monomers from apple cutin for these strains. The detergent n-octyl glucoside stimulated the cutinase activity of culture fluids from strains K799 and DAR 41352, but not from strain 1499A. Temperature effect studies done at 22, 28 and 37 C (the optimal for growth) indicated that esterase/cutinase production was most rapid at 28 and 37 C for strains K799 and DAR 41352 with highest levels achieved at 28 C for K799 and

22 C for DAR 41352. Enzyme activity was relatively low at all three temperatures for strain 1499A with highest activities seen at 22 and 28 C.

DISCUSSION

The availability of a simple spectrophotometric assay for general esterase activity coupled with the development of a novel HPLC technique for separation, identification and quantification of cutin monomers in our laboratory allowed us to conveniently screen a large collection of bacteria for extracellular cutinase production without the need for radiolabelled cutin. The results using the conditions employed indicate that the ability to hydrolyze the plant biopolyester cutin is not widespread among plant-associated bacteria even though all three previously reported cutinase-producing bacteria are plant-associated. However, the use of other cutins and culture media may lead to the identification of additional cutinase producers in our collection. The importance of bacterial cutinase production for phytopathognicity and/or epiphytic survival is not known.

Production d'enzymes de dégradation de la cutine par des bactéries pathogènes

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Une collection de plus de 277 souches bactériennes filamenteuses et non filamenteuses a été criblée pour leur activité cutinasique, enzyme de dégradation de la cutine des plantes (polymère d'acide gras hydroxylés liés entre eux par des liaisons esters). Les souches testées englobent les bactéries saprophytes et pathogènes. Les bactéries ont été cultivés sur différents milieux liquides avec de la cutine de pomme comme unique source de carbone. L'activité estérasique extracellulaire a été déterminé par spectrophotométrie avec le butyrate de p-nitrophényle. Les cultures avec un activité estérasique élevée inductible par la cutine ont été testés pour leur activité cutinasique. L'identité des acides gras hydroxylés libérés a été confirmé par chromatographie gazeuse couplée à la spectrométrie de masse alors que l'analyse quantitative a été realisé par

chromatographie liquide haute performance couplée à un détecteur massique, technique développée dans notre laboratoire. A l'aide de cette méthode d'analyse, nous avons pu ainsi découvrir 4 souches positives de *Streptomyces* spp. et 3 souches positives de *Pseudomonas aeruginosa*. Deux souches sur 7 présentent une augmentation de leur activité cutinasique par addition de n-octyl glucoside dans le milieu réactionnel. Les températures optimales de production de ces activités cutinasiques sont inférieures à 37 C pour les souches de *Pseudomonas aeruginosa* .

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A study on the correlation between the pathogenicity and the synthesis of *Xanthan* in mutant strains of *X. campestris*

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ABSTRACT

The biosynthesis of *Xanthan* is a main taxonomic character of *X. campestris*. Different mutant strains of *X. campestris* were isolated insensitive to bacitracin and rifamycin differing from main (wild) one belonging to the other pathovars. The studied mutants, *Xcc*9, *Xcv*12, *Xcc*10, *Xcc*11, *Xcg*118, *Xcf*19 did not synthesize *xanthan* and the mutants *Xcg*17 and *Xcg*18 were avirulent.

INTRODUCTION

X. campestris are gram-negative phytopathogenic bacteria. Taxonomically *X. campestris* is devided into pathovars, mainly according to the host. The synthesis of exopolysaccharide is main taxonomic character of *X. campestris*. One of the products of *X. c.* that is thought to contribute to pathogenicity is its extracellular polysaccharide. Plugging of xylem plant tissue appears to be caused by synthesis of *xanthan* by *X. campestris* since it is found at the site of the lesion (SUTTON and WILLIAMS, 1970). However, a direct link between the ability to synthesize *xanthan* and pathogenicity is not yet establlished.

It was supposed that some relationship exists between the pathogenicity of *X. campestris* and the synthesis of pectolytic enzymes (STARR and NASNO, 1967) and low molecular weight acidic phytotoxic substances (NODA *et al.*, 1980 ; PERREAUX *et al.*, 1982), but no evidence has been presented for a role of these latter substances in disease.

Some data exist about the importance of extracellular protease synthesized by *X. campestris* for the virulence of different pathovars (DOW *et al.*, 1990).

In the present study we describe some mutants differing in their pathogenicity and inability to synthesize EPS.

MATERIAL AND METHODS

Micro-organisms

We obtained two *Xanthomonas campestris* strains from Plant Protection Institute - Kostinbrod, Bulgaria :

- *X. c.* pv. *vesicatoria* H-58
- *X. c.* pv. *glycines* H-191

Strains : *X. c.* pv. *campestris* ITS-342 and *X. c.* pv. *phaseoli* T-13 were isolated previously in laboratory "B-4", Institute of Bioproducts, Bulgaria. The strains were maintained on YM-agar. They were transferred every two weeks to maintain good viability.

Mutagenesis of *X. campestris*

About 2x10 freshly grown cells (A600 of 2) were suspended in 2 ml of minimal salts medium and shaken at 30°C with 100-250 µg/ml nitrosoguanidine for 2 to 5 hrs. Samples of 0.5 ml were taken from each treatment, washed twice with YM medium, suspended in 2 ml medium diluted and plated on YM-agar with 1% glucose and 250 µg/ml bacitracin to screen for the appearance of non-mucoid colonies. Single rifamycin or bacitracin resistant colonies were transferred to "master" plates with sterile toothpicks to give an array of 50 colonies per plate, which were replicated with velvet to plates of minimal medium 1 (MILLER, 1972), minimal medium 2 supplemented with Casamino acid and medium 3 YM-agar with 1 x (w/v) glucose. The use of these media permitted detection of auxotrophs (medium 1 and 2) and polysaccharide secretion (medium 3).

Pathogenicity test

The pathogenic ability of the mutants was tested .

1- by hypersensitive reaction (HR) on tobacco (KLEMENT, 1963)

2- by the vacuum infiltration method on the test plants tomato, peper, soja and bean (BOGATZEVSKA, 1988)

RESULTS AND DISCUSSION

Isolation of mutants of *X. campestris*

X. campestris mutants defective in production of extracellular polysaccharide were obtained by chemical mutagenesis of strains H58, H191, ITS-342 and T13

with nitrosoguanidine. After growth at 30°C for three days on YM-agar, non-mucoid colonies were selected and purified for further use.

The mutants characterized in this report differ in morphology, sliminess and size of the colonies, degree of pigmentation, resistance to Bacitracin (Bac.). From the studied four strains are isolated slime and non-slime mutants. All mutants were resistant to Bac. The parent strains were sensitive to Bac. Most of Xgs^- mutants were auxotrophic. All Xgs^- mutants from different pathovars were cultivated on minimal medium containing glucose, fructose and mannose.

Pathogenicity test

The studied strains showed great pathogenic variability (table 1). The mutants 342m3 and 342m5 from the parent strain ITS-342 showed increased pathogenicity to plants as soybeans and beans, whereas the other obtained mutants infected these plants at a low degree. The parent strain did not show the same pathogenicity. The mutants like 342m1, 342m4, 342m6 and 342m11 caused HR on tomatoes and induced some spots, in contrast to the other mutants from strain ITS-342. The mutants 58m1, 58m2, 58m6 of strain H58 showed extremely high pathogenicity to soybean and bean, whereas the parent strain was not pathogenic to soybean. The performed studies indicated that 58m1 did not synthesize exopolysaccharide, the other mutants produced EPS. All mutants lost pathogenicity to tomatoes and pepper.

Alteration in pathogenicity were also observed in *Xc*pv glycines - H191. Both mutants did not show HR to tobacco. At the same time they developed pathogenicity to bean in contrast to the parent strain and lost pathogenicity to tomatoes. Studying strain *Xc*pv *phaseoli* T13 and its mutant , we observed that the mutant loose its pathogenicity to tomatoes, but appeared to be pathogenic on pepper. At the same time, mutant 13m1 did not synthesize EPS.

Summerizing all the results obtained, it could be concluded that the pathogenicity did not correlate with the synthesis of EPS. According to SUTTON (1970), EPS has a pronounced affect on the pathogenicity, but our results did not confirm these reports. We assume that EPS is maybe connected with development of the later period in disease.

The obtained data on pathogenicity of mutants in comparison with the parent strains seemed to indicate that the straingent differentiation of the different pvs of the group of *X. campestris* is impossible.

The future investigations shall deal with genetics of the mutants and their mechanism of the pathogenicity.

Table 1. Bacterial strains and mutants

Strains or mutants	Relevant characteristics	Strain or mutants	Relevant characteristics
X.c.pv. campestris ITS-342	wild type , Xps+	X.c.pv.vesicatoria H58	wild type, Xps+
342m1	Xgs+,prototroph,Bac	58m1	Xgs-,auxotroph,Bac
342m2	Xgs+,prototroph,Bac	58m2	Xgs+,prototroph,Bac
342m3	Xgs+,prototroph,Bac	58m3	Xgs-,auxotroph,Bac
342m4	Xgs+,prototroph,Bac	58m4	Xgs-,auxotroph,Bac
342m5	Xgs+,prototroph,Bac	58m5	Xgs-,auxotroph,Bac
342m6	Xgs+,auxotroph,Bac	58m6	Xgs+,prototroph,Bac
342m7	Xgs+,auxotroph,Bac	X.c.pv.glycines H191.	wild type, Xps+
342m8	Xgs+,auxotroph,Bac	H191	
342m9	Xgs-,auxotroph,Bac	191m1	Xgs+,prototroph,Bac
342m10	Xgs-,auxotroph,Bac	191m1	Xgs+,prototroph,Bac
342m11	Xgs-,auxotroph,Bac	X.c.pv.phaseoli T13	wild type, Xps+
		13m1	Xgs-,auxotroph,Bac

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A model for mechanisms of resistance and susceptibility on a molecular level in plant - microbe-interactions

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Summary

A model is proposed depicting the interaction of bacterial extracellular polysaccharides (EPS) with cell wall polymers (agglutinins) of susceptible and resistant plants. The model comprises the phenomenon of cultivar-race-specificity.
In the compatible interaction agglutinin with low content of galacturonic acid and low agglutinating activity is extruded into the intercellular space in small amounts and forms a synergistical gelling-system with alginate-rich EPS from highly virulent strains, thus preventing the bacteria from resistance reactions (recognition, agglutination) of the plant. Extracellular bacterial lipopolysaccharides inactivate the agglutinin by precipitation.
In the incompatible interaction galacturonic acid-rich, highly active agglutinin precipitates the bacterial alginate. The agglutinin reacts with the bacterial surface-LPS of the unprotected bacteria, thereby agglutinating them.

keywords: molecular plant-microbe-interactions, agglutinin, extracellular polysaccharides, lipopolysaccharides, *Pseudomonas syringae* pv. *phaseolicola*

Introduction

Bush bean (*Phaseolus vulgaris*) leaves inoculated by *Pseudomonas syringae* pv. *phaseolicola* were selected as a model for leaf spot diseases caused by pseudomonads. Earlier, a correlation between the degree of resistance to *P.s.* pv. *phaseolicola* and the intensity of agglutination by extracts from different bush bean cultivars *in vitro* was observed (El-Banoby and Rudolph 1980a, 1981a). We therefore tried to characterize the molecular basis for the differential interaction between polymers of bacterial and plant origin.

Material and methods

The agglutinin was extracted from the first trifoliates of *Phaseolus vulgaris* cv. Red Kidney -RK- (susceptible to *P. phaseolicola*), cv. Red Mexican -RM- (susceptible to *P. phaseolicola* race 2, resistant against race 1) and breeding line "O2" (resistant), as described by El-Banoby and Rudolph (1980a). The homogenate in 0.1 N HCl was centrifuged and the supernatant precipitated by 38% ammonium sulphate. The supernatant fraction contained the agglutinin.
For inoculation of plants and agglutination tests the following strains of

P. phaseolicola from the GSPB (Göttinger Sammlung Phytopathogener Bakterien) were used: 707 (Ro), 595 (CH) (race 1); 615 (FV), 1715 (N7) (race 2); *Pseudomonas putida, Pseudomonas fluorescens* (saprophytes). The bacteria were harvested from 100 ml-cultures grown in Keen and Williams (1971) medium. Extracellular polysaccharides (EPS) and lipopolysaccharides (LPS) were produced and prepared according to Wydra (1991), Groß and Rudolph (1987a,b) and Westphal and Jann (1965), respectively.
For agglutination and precipitation assays 10 μl of agglutinin solution in different dilutions were mixed with 10 μl of bacterial suspension (1.5×10^{10} cfu/ml) or EPS/LPS suspension in Petri dishes. Agglutination/precipitation were scored as degree of flocculation (scale 1 to 4) under the dissecting microscope.
Hexoses, uronic acids and protein contents were quantified according to Dubois et al. (1956), Bitter and Muir (1962) and Bradford (1976), respectively. Viscosity of EPS was measured with a falling ball viscosimeter (Haake, Germany).

Results

Molecular interactions

Lipopolysaccharides (LPS) from *P.s.* pv. *phaseolicola* and the saprophytes *P. fluorescens* and *P. putida* precipitated stronger with agglutinin from the resistant line than with agglutinin from the susceptible cv. (Wydra and Rudolph 1990). The addition of EPS, alginate, sodium-polygalacturonate and buffer with pH>5 inhibited the precipitation, while increasing ionic strength and addition of lectins increased the reaction, and fucoidan (highly anionic polysaccharide) had no influence. These results indicate a possibly specific polysaccharide-polysaccharide-interaction.

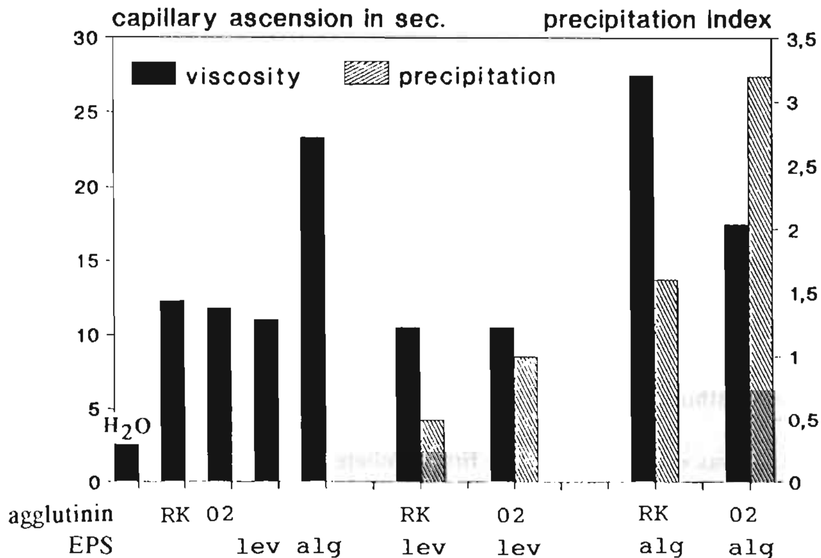

Fig. 1: Viscosity and precipitation after mixing of levan-rich (lev) or alginate-rich (alg) EPS with agglutinin of the susceptible (RK) and the resistant cultivar ("02")

Mixing of alginate-rich EPS with agglutinin from cv. RK (compatible interaction) resulted in increased viscosity. In the incompatible interaction agglutinin from the line "02" precipitated stronger with alginate-rich EPS than did agglutinnin from cv. RK (compatible interaction) (fig. 1), and the degree of precipitation by 25 strains was positively correlated with the proportion of alginate and protein in the EPS (data not shown). Viscoelasticity increased after mixing alginate-rich EPS with agglutinin from both cultivars. The lowest concentration of agglutinin from the resistant cultivar to agglutinate 10^{11} bacteria/ml was 125 μg agglutinin/ml.

Analysis

The purified agglutinin contained 80% total sugar, 15% uronic acids and about 5% protein. Chromatography on DEAE fractogel separated a neutral fraction, containing mainly the cell wall sugars arabinose and galactose and small amounts of rhamnose and glucose, and an acidic fraction, which contained in addition galacturonic acid (fig. 2). The acidic fraction showed higher agglutinating activity. The agglutinin of the resistant cultivar contained more galacturonic acid than the agglutinin of the susceptible cultivar. Depolymerization of the agglutinin with pectinesterase and polygalacturonase decreased the agglutinating activity and thereby revealed a possible importance of polgalacturonic acid for the activity of the agglutinin (Wydra 1991). After inoculation of bean leaves with *P.s.* pv. *phaseolicola* the agglutinin of both cultivars contained higher proportions of galacturonic acid.

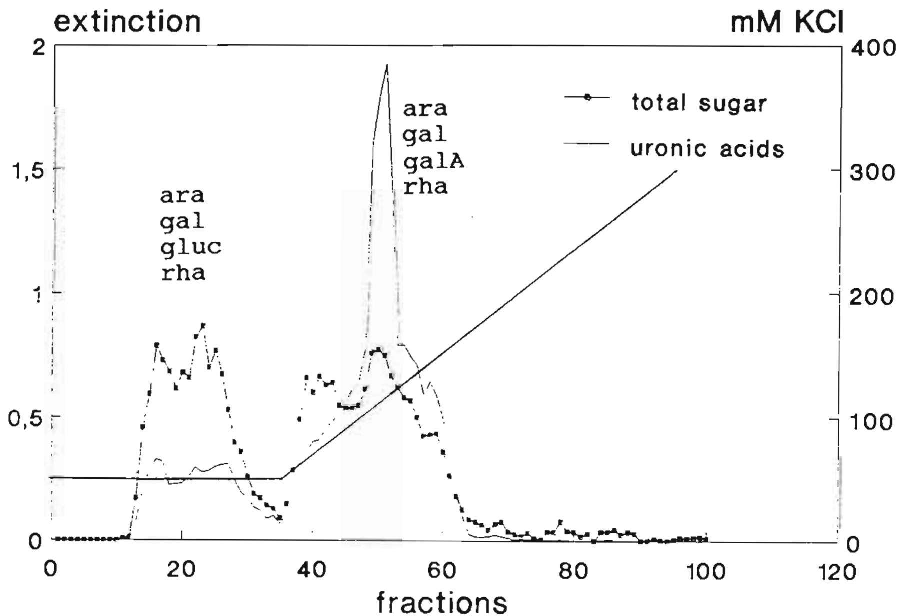

Fig. 2: Column chromatography on DEAE fractogel of agglutinin from the resistant line "02"

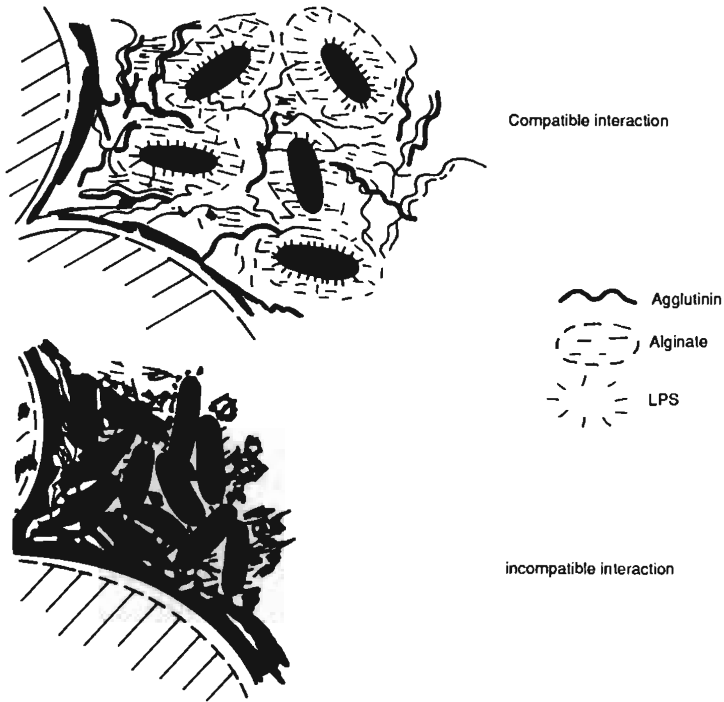

Fig. 3: Model for the interaction of bacterial surface polymers with plant cell wall polymers in the compatible and incompatible interaction

In the compatible interaction with typical symptoms of water-congestion, alginate-rich EPS from highly virulent strains and agglutinin are supposed to synergistically form a highly viscous gel, which increases the water-holding capacity of the EPS, thus creating a favourable environment for bacterial multiplication and preventing the bacteria from the resistance reactions of the plant (recognition, agglutination). The agglutinin has a low activity and is released in low quantities into the intercellular space. Extracellular bacterial LPS precipitate the agglutinin, resulting in its inactivation (fig. 3).

During incompatibility and absence of water-soaking the interaction between EPS and agglutinin decreases the gel-forming capacity of the EPS by precipitation (El-Banoby et al. 1981). The supply of the bacteria with water and nutrients is restricted, and the bacteria are unprotected against the resistance reactions of the plant in the intercellular space. Recognition events of the plant are provoked by contact of the extracellular LPS with the plant cell wall. Active, galacturonic acid-rich agglutinin reacts with the bacterial surface-LPS and thereby agglutinates the bacteria. This is supposed to be one of the first steps in the sequence of resistance reactions.

Discussion

The here reported inhibition of precipitation between LPS and agglutinin by bacterial alginate-rich EPS as well as by Na-polygalacturonate suggests a specific interaction beween agglutinin and LPS and a role of polygalacturonate for the activity of the agglutinin. The inhibition experiments (pH-shifts, no inhibition by fucoidan) reveal a not-electrostatic, possibly specific polysaccharide-polysaccharide-interaction as basis for the reaction between the bacteria and the plant cell wall substances, depending on the qualitative composition of the agglutinin as well as the EPS.

The interaction, which depends on the composition of the polysaccharides (degree of intermolecular association, number and composition of side chains of the polysaccharide backbone) can lead to the formation of either precipitates or gels (Wydra and Rudolph 1992). A synergistical gelling-system between alginate from algae and pectic substances has been demonstrated by Morris et al. (1982). The increase in viscosity during interaction of products from compatible partners may be decisive during pathogenesis in enabling the bacteria to colonize the intercellular space (Rudolph et al. 1989). A cultivar-race-specificity can be assumed: the race-specific production of alginate with differences in amount and viscosity of the alginate can result in differences in the suitability for gel-formation with the agglutinin of the susceptible plant.

The analysis of the agglutinin revealed the typical composition of a pectic polysaccharide, namely an arabinogalactan, probably containing side chains with varying amounts of neutral or acidic sugars. The elution profile is typical for pectic polysaccharides (De Vries et al. 1986). Only the loose, water-soluble portion of the pectic cell wall polysaccharides was extracted. In contrast Slusarenko and Wood (1981) extracted an agglutinin, differing from the here described one, with a high content of galacturonic acid. The polysaccharides arabinogalactan and rhamnogalacturonan may be parts of a single complex of pectic polysaccharides (O'Neill et al. 1990).

The model comprises an explanation for molecular interactions in the compatible and incompatible reaction. While in the compatible interaction the virulence factors of the bacteria - alginate and extracellular LPS - inhibit the resistance reactions of the plant, in the incompatible interaction a highly active, galacturonic acid-rich agglutinin released from the cell wall in higher amounts into the intercellular space, precipitates the bacterial alginate and agglutinates the bacteria, thus restricting their multiplication.

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Exopolysaccharides of phytopathogenic pseudomonads

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SUMMARY

A total of 48 strains of phytopathogenic pseudomonads were screened for the ability to produce the acidic exopolysaccharide (EPS) alginate in mineral liquid media. Most of *Pseudomonas syringae* pathovars when grown on gluconate medium produced alginic acid (as detected by thin layer chromatography). However, when sucrose was the primary carbon source during culture *in vitro*, the tested organisms produced also levan (a fructan).

Out of 26 tested *P. syringae* strains isolated from the family Umbelliferae, specially the annual herb coriander (*Coriandrum sativum L.*), 15 isolates produced high amounts of EPS (3-5.5 g/l, compared with *P.s.*pv. *phaseolicola* N7, 1.9 g/l).

When the EPS from coriander isolates was stored for one month a decrease in viscosity of 20-80 % occurred.

EPS from culture filtrates of 3 different pathovars were obtained by ultrafiltration. The preparations induced persistent water-soaked spots when infiltrated into leaves of a halo-blight susceptible bush bean cv. as host plant. Weakly persistent spots were obtained on the susceptible cv. as non-host plant. No persistent water-soaking was induced on resistant plants.

INTRODUCTION

Many phytopathogenic bacteria are known to produce exopolysaccharides (EPS) *in vitro*. Previous studies indicated that *Pseudomonas syringae* pathovars when grown on a variety of carbon sources *in vitro* can produce alginic acid (a linear backbone consisting of varying ratios of mannuronic and guluronic acids). However, when sucrose is present as the primary carbon source during culture *in vitro* these

bacteria produced levan (a fructan), alginic acid, or both levan and alginic acid (Fett et al. 1986; Gross and Rudolph 1987).
Infiltration of purified EPS into host tissues is reported to induce a variety of physiological host responses, including induced water soaking of leaf tissues (El-Banoby and Rudolph 1979). However, polyfructans and alginates did not induce prolonged water soaking of soybean leaf tissues (Fett et al. 1986).

MATERIALS AND METHODS

Bacterial strains: From the institute's collection (GSPB).
Production of EPS: All strains were grown on gluconate mineral medium supplemented with 0.05 % sodium desoxycholate and 0.1 mM potassium phosphate buffer (Wydra 1991). Selected strains were also tested for acidic EPS production on sucrose mineral medium (modified after Dorn et al. 1974).
Preparation of EPS & thin layer chromatography (TLC) : We followed the methods described by Gross and Rudolph (1987).
Viscosity was measured in the culture filtrate with a falling ball viscosimeter.
Bioassay : The ability of EPS preparations to induce persistent water-soaking in plant leaves was determined (Rudolph 1978).

RESULTS

Screening for alginate-positive strains

A total of 48 strains of phytopathogenic pseudomonads were screened for the ability to produce the acidic exopolysaccharide (EPS) alginate in gluconate mineral medium (Table 1). Most of the strains isolated from the family Umbelliferae produced high quantities of EPS, which often contained high proportions of alginate. The viscosity of some of these EPS preparations was remarkably high. However, after a storage period of 1 month at 25°C these lyophilized EPS preparations lost 20-80% of viscosity. On the other hand, strain N7 of *P.s.*pv.*phaseolicola* produced EPS with highly stable viscosity.
The only predominant monosaccharide detected by TLC in the EPS hydrolysates of most of the tested strains was mannuronic acid (manA) after growing the bacteria in gluconate medium.Strains from the pathovars *phaseolicola* (N7), *coriandricola* (1,5,11, 34,39,41,zu 4a,zu K7c) and *P.syringae* (W42 isolated from carrot) proved to be high producers of alginate. In contrast, strains isolated from fennel showed low or no proportions of manA, and all *P. marginalis* and *P. viridiflava* strains were alginate-negative. Fructose and rhamnose were not detected when gluconate was the sole carbon source in the medium. Only when the bacteria were grown on sucrose, the EPS of pv. *phaseolicola* and *glycinea* contained fructose.

Table 1: Production and composition of exopolysaccharides (EPS) produced by several phytopathogenic pseudomonads grown in liquid gluconate medium.

Species/ pathovar	strain	Viscosity mPa.s	EPS g/l	manA
Pseudomonas syringae				
pv. *aptata*	Z 84-1	1.2	0.79	+
pv. *atrofaciens*	5-1	1.3	0.98	++
pv. *glycinea*	GSPB 1201	1.4	1.07	++
	GSPB 1203	1.2	1.13	+
	SB1a	1.7	2.38	++
	Baron a	1.3	2.06	++
	4B2b Pol	1.3	2.41	+
	Dawson a	1.6	2.75	++
	Dorado a	1.3	2.01	++
	H-7a	1.2	2.44	+
	H-2c	1.2	1.83	+
pv. *lachrymans*	Bamya II	3.5	1.99	+
	La w	1.2	1.40	+
pv. *phaseolicola*	N7	4.3	1.88	+++
	847	1.2	1.56	+
	1409	1.2	0.87	+
	1643	1.2	1.35	+
pv. *tomato*	Mav 2s, tom 2schr	1.2	0.94	+
	Mav 2s, tom sl	1.2	0.92	+
	Mav 8s	1.2	1.44	+
pv. *coriandricola*	1	15.4	3.79	+++
	3	21.3	4.53	++
	5	5.4	2.54	+++
	9	16.5	5.09	+
	11	44.0	4.57	+++
	21	21.0	5.48	++
	32	5.8	3.71	++
	34	10.2	3.71	+++
	35	9.8	3.40	+
	36	3.9	4.30	+
	39	33.1	3.36	+++
	41	7.6	4.16	+++
	NCPPB 3115	3.0	2.70	+
	Zu 4a	8.4	3.11	+++
	Zu K7c	9.6	3.91	+++
	Bi 4.2	1.2	0.59	+
	H4	1.2	0.40	++
Pseudomonas spp				
from *Anthriscus silvestris*	W4	1.2	1.11	+
from *Daucus carota*	W42	6.7	3.16	+++
	W43	14.1	5.15	++
from *Foeniculum vulgare*				
(Ia)	GSPB 1765	1.2	1.35	+
(Ia)	GSPB 1766	1.2	0.24	+
(Ib)	GSPB 1767	1.2	1.19	+
(II)	GSPB 1768	1.2	0.53	-
(IV)	GSPB 1762	1.2	1.61	-
(II)	GSPB 1764	1.2	0.45	-
Pseudomonas marginalis	DSM 50276	1.3	1.44	-
Pseudomonas viridiflava	NCPPB 635	1.2	0.96	-

The predominant sugars were roughly quantified on TLC chromatograms by visual comparison of spot size and colour intensity (+++/ ++/ +) = high/ medium/ low proportion, (-) = not detected, manA = mannuronic acid (the detected monomer in hydrolysates)
Note : rhamnose and fructose were not detected by TLC.

Persistence of water-soaked lesions

EPS were partially purified from three bacterial strains (*P. syringae* pv. *phaseolicola* N7, pv. *coriandricola* no. 21 and *P. syringae* W43) and infiltrated into trifoliates of two bush bean cultivars at a concentration of 10 mg/ml (Table 2). Some water-soaked lesions induced by EPS preparation from *P.s.*pv. *phaseolicola* N7 on the halo-blight susceptible cultivar "Red Kidney" persisted for 6 days. Fewer water-soaked spots persisted for 2 and 4 days after infiltration of EPS preparations from *P. syringae* W43 and *P.s.*pv. *coriandricola* 21, respectively. On the other hand, none of the EPS-preparations produced water-soaking in the breeding line "02" which is resistant to *P.s.*pv. *phaseolicola.*

Table 2: Number of persistent water soaked spots in trifoliates of bush bean (*Phaseolus vulgaris* L.) after infiltration of various bacterial EPS preparations

	Cultivar "Red Kidney"				Breeding line "O2"			
tpi	H_2O	N7	COR.21	W43	H_2O	N7	COR.21	W43
	s+w	s+w	s+w	s+w	s+w	s+w	s+w	s+w
0 h	20	20	20	20	20	20	20	20
18 h	14	9+5	16	14	5	8+1	4	6
24 h	4+1	5+3	10	9+2	5	7+1	1	3+2
36 h	1	5+3	7	9+1	5	4+1	0	3+2
42 h	0	0+3	0+2	0+3	5	0	0	0+2
48 h	0	0+2	0+2	0+3	2+1	0	0	0+2
3 days	0	2+1	0+2	0	0	0	0	0
4 "	0	5+1	0+2	0	0	0	0	0
5 "	0	4+1	0	0	0	0	0	0
6 "	0	4+1	0	0	0	0	0	0
			Removal of plastic bags					
7 "	0	1+1	0	0	0	0	0	0
8 "	0	1+1	0	0	0	0	0	0

tpi : time after infiltration
N7 : EPS preparation from *P.s.*pv.*phaseolicola* N7
COR.21 : " " " *P.s.*pv.*coriandricola* 21
W43 : " " " *P.syringae* W43 "from carrot"
s : strong spot
w : weak spot
Red Kidney : susceptible to *P.s.*pv. *phaseolicola*
02 : resistant against *P.s.*pv.*phaseolicola*

DISCUSSION

These studies showed that, under certain appropriate culture conditions, many strains of phytopathogenic pseudomonads are capable of synthesizing acidic exopolysaccharides (alginate), although amounts and viscosity varied considerably between the isolates.

An alginate-like polysaccharide produced by a phytopathogenic bacterium (*P.s.*pv.*phaseolicola*) has first been described by Gross and Rudolph (1984). Likewise, Osman and Fett (1985) demonstrated alginic acid in the EPS of *P.syringae* pathovars *glycinea*, *morsprunorum*, *papulans*, *phaseolicola*, and *tabaci*. Other bacterial species producing alginate are the N-fixing *Azotobacter vinelandii* (Gorin and Spencer 1966) and human pathogenic *P. aeruginosa* strains (Evans and Linker 1973). Mutants of *P. fluorescens*, *P. putida*, and *P. mendocina* were also reported to exhibit this capacity (Govan et al. 1981).

Although in our studies a complete chemical characterization of EPS composition was not achieved, they all appear to be polymers of mannuronic acid as detected by TLC after growing the bacteria in gluconate mineral medium. Levan was only synthesized with sucrose as primary carbon source. Our results coincide with the results reported by Gross and Rudolph (1987).

In earlier studies, (Rudolph 1978; El-Banoby 1980) permanent water-congestion was only caused in susceptible bean leaves, but not or only weakly in resistant ones and not at all in non-host plants. Our experiments showed, however, that also in the halo-blight susceptible bean cv. "Red Kidney" as non-host, weakly persistent water-soaked spots were obtained.

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Structure and phytotoxic activity of *Clavibacter (Corynebacterium) sepedonicum* extracellular polysaccharide

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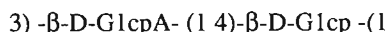
ABSTRACT

The acidic extracellular polysaccharide from *Clavibacter (Corynebacterium) sepedonicum*; isolated by ion-exchange chromatography on DEAE-TSK 650 M gel, contains equal amounts of D-glucose and D-glucuronic acid. Results of periodate oxidation, ^{1}H- and ^{13}C-n.m.r. analysis data led to the following structure of the polysaccharide:



3) -β-D-GlcpA- (1 4)-β-D-Glcp -(1

Introduction of this polysaccharide into tomato plant cuttings resulted in the loss of turgor in leaves or branches.

KEYWORDS
Extracellular polysaccharide, phytotoxic activity, *Clavibacter (Corynebacterium) sepedonicum.*

In addition to cell-wall polysaccharides phypathogenic coryneform bacteria produce extracellular glycopolymers which may be responsible for plant wilting symptoms. But studies concerning the investigation on phytotoxic substances mainly contain data about their biological activity. The chemical nature of toxicity and virulence factors was not identified until now. Since there is a correlation between structure and type of reactions induced by polymers in living cells it's necessary to study the chemical structure of causative factors of certain diseases.
The investigators believe that extracellular glycopolymers are one of the compounds preventing the induction of plant protective reactions.
We have made comparative studies on composition, structure and phytotoxic activity of glycopolymers produced by *Clavibacter (Corynebacterium) sepedonicum* causing wilting

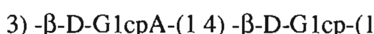
activity of potato plants. The investigations on phytotoxic activity of the culture filtrates of five strains of *C. sepedonicum* permitted to decide in favour of two strains. The culture filtrate of these strains are characterized by phytotoxic activity in tests with seeds of cress, wheat, corn and cucumbers. The culture filtrates of the strains investigated were freeze-dried and purified by ion-exchange chromatography on DEAE-TSK gel. As a result three fractions were obtained: the first - neutral (GP 1) and the next two - acidic (GP 2 and GP 3).

Studies with plant cuttings revealed that the neutral glycopolymers didn't show wilt inducing activity - the first and predominant symptom after infection. Introduction of acidic polysaccharides into tomato plant cuttings resulted in the loss of turgor in leaves or branches.

GP 3 of C. *sepedonicum* showed the highest wilt inducing activity.

Chemical identification indicated that glucose is a predominant neutral monosaccharide for all glycopolymers investigated and in GP 3 glucose is the only neutral monosaccharide. We studied the structure of this glycopolymer using chemical methods such as methylation, periodate oxidation and also physical methods - nuclear magnetic resonance spectroscopy (n.m.r.).

^{13}C-n.m.r. spectrum (Fig.1) showed that the polymer has a regular structure with disaccharide repeating units containing one hexose - glucose and one glucuronic acid. From the analytical data received by chemical and physical methods we concludet that the extracellular polysaccharide inducing wilting on tomato plant cuttings had the following structure:



3) -β-D-GlcpA-(1 4) -β-D-Glcp-(1

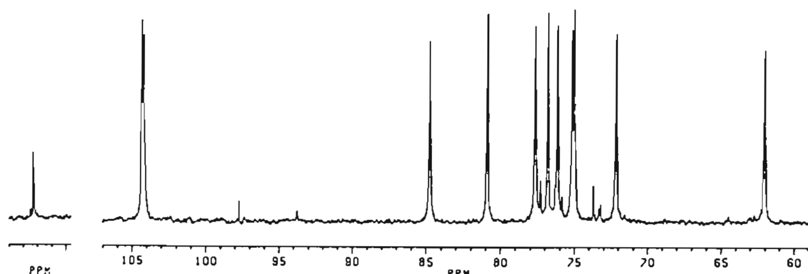


Fig. 1. ^{13}C-n.m.r. spectrum of *C. sepedonicum* extracellular polysaccharide

This polysaccharide consists of disaccharide repeating units composed of residues of D-glucopyranose and D-glucuronic acid connected by β-1,3 and β- 1,4 linkages.
It's necessary to indicate that toxins of phytopathogenic corynebacteria were studied very poorly. Until the present time the structure of only one toxin isolated from *C. insidiosum* was determined. The authors (GORIN & SPENCER, 1980) showed that the phytotoxin appeared to be a glycopeptide, the polysaccharide part of which consists of tetrasaccharide repeating units composed of one D-galactosyl, one D-glucosyl and two L-fucosyl residues. Pyruvic acid as ketal cycle is linked to C4 and C6 of D-galactosyl residues. Ries and Strobem (1970) isolated a phytotoxic glycopeptide with L-fucosyl, D-glucosyl and D-galactosyl residues with ketodeoxy-glucuronic acid as acidic component from other strain of *C. insidiosum*. L-fucose was a predominant neutral polysaccharide. On the basis of the data received some researchers proposed that fucose appeared to be a peculiar marker for the bacterial toxin of glycopeptide nature. But studying the composition of phytotoxic glycopeptide of *C. michiganense* (RAI & STROBEL, 1969) the presence of small amounts of fucose was shown while glucose and mannose were predominant monosaccharides. Our results on the chemical identification of *C. sepedonicum* phytotoxin also indicate glucose to be a predominant monosaccharide of the investigated extracellular polysaccharide. Peptide parts in this polysaccharide are absent. This is in agreement with the results of other authors (VAN DEN BULK *et al.*, 1989) who reported that a phytotoxic high molecular weight fraction of *C. michiganense* contained 93% glucose and 0.3-0.4% protein. These authors in further studies planned to purify and characterize the high molecular weight fraction. However we didn't find results on the chemical characterization of this fraction in the available literature.
Which chemical determinants are responsible for biological activity of phytotoxins? Researchers reports on acid lability of substances investigated. Only pyruvic, 2-keto-deoxyoctonic or uronic acids are unlikely to be responsible for phytotoxic activity because these components are detected in polysaccharides of other bacteria which do not show phytotoxic activity. It's more likely that acidic components favor the creation of such conformations of glycopolymers which are responsible for phytotoxic action. It's also possible that the nature of linkage between the individual monosaccharide is important in phytotoxic activity of these polysaccharides.
Comparative structural analysis of two glycopolymers exerting phytotoxic activity (*C. insidiosum* and *C. sepedonicum*) showed the presence of analogous linkages β-1,3 and β-1,4.
Therefore on the basis of results of our own investigations and also literature data we propose that substances with different chemical composition and structure fulfill similar

biological function - to induce wilting of plants. It may be supposed that there is biological but not chemical determination of our understanding of a phytotoxin.

ACKNOWLEDGMENT

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Structure and functions of lipopolysaccharides from *Pseudomonas syringae* (serogroup II)

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ABSTRACT

Lipopolysaccharides (LPS) from strains of *P. syringae* pv. *syringae* 90a, 435, 467, pv. *atrofaciens* K-1025, pv. *morsprunorum* CF-4 referred by PASTUSHENKO and SIMONOVITCH (1979) to serogroup II have been studied. The strains were shown to be heterogeneous by chemical composition of core and lipid A and structure of O-specific polysaccharide. The preparations heterogeneity in serological cross reactions was also detected. O-specific polysaccharides of the strains having similar structures were not identical in serological tests. The supposition on lipid A role in serogrouping of *P. syringae* strains have been advanced.

Only intact LPS preparations caused specific symptoms after injection into the plant.

KEYWORDS

Lipopolysaccharide, core oligosaccharide, O-specific polysaccharide, lipid A, function of Lipopolysaccharides, *P. syringae*.

MATERIALS AND METHODS

Lipopolysaccharides of phytopathogenic bacteria attract increasing interest of scientists in many countries (England, USA, Japan, etc.). It is due to the finding LPS of microbial cell are characterized by a unique semi-functionality, whereas in phytopathogenic bacteria the composition, structure and functions of these biopolymers have been studied insufficiently.

At the Institute of Microbiology and Virology for many years the objects of study were LPS from *Pseudomonas syringae*, the members of the various serogroups of classification scheme elaborated by PATUSCHENKO and SIMONOVITCH (1979) for the determination of chemical bases for their serogrouping, determinants of antigenic specificity and elucidation of their role in pathogenesis.

The given report presents data on LPS of *P. syringae* strains belonging to serogroup II. These are *P. syringae* pv. *syringae* (*P. cerasi*) 435 and 467, pv. *morsprunorum* CF-4 - the agents of tree forest and fruit cultures ; pv. *syringae* (*P. holci*) 90a and pv. *atrofaciens* K-1025 - the incitant of diseases of cereals.

Using methods we have developed earlier (ZDOROVENKO *et al.*, 1982 ; KNIREL *et al.*, 1986) LPS preparations characterized by high carbohydrate content and low protein content have been obtained.

As it is known LPS consists of three components : lipid A, core oligosaccharide (core) and O-specific polysaccharide (O-PS) which differ by composition and structure.

For obtaining separate parts of the LPS macromolecule the mild acidic hydrolysis has been carried out and as a result, lipid A was obtained. After gel-filtration of the carbohydrate part of the molecule on column with Sephadex G-50 the core oligosaccharide and O-specific polysaccharide have been obtained (KNIREL *et al.*, 1986).

The analysis of lipid components demonstrated that they contained fatty acids with chain length of carbon atoms from C_{10} to C_{18} (Table 1). In their composition there were saturated, unsaturated fatty acids and oxyacids. All strains revealed 3-oxydodecanic ($3OH\text{-}C_{12:0}$) fatty acid. This acid is a marker of pseudomonad lipid A.

Table 1 : Fatty acid composition of lipid A

Strain	Acids						
	30 H C10:0	C12:0	20H C12:0	30H C12:0	C16:1	C16:0	C18:1	C18:0
CF-4	5,7	14,9	10,0	8,7	22,8	21,7	13,6	2,1
90a	-	0,5	4,2	5,2	25,5	33,7	25,2	3,4
435	2,3	6,6	6,1	6,4	29,6	23,3	22,7	3,2
467	5,1	14,9	9,7	5,7	0,7	15,1	22,3	1,1
K-1025	1,5	3,9	5,9	4,9	27,6	33,4	17,1	2,6

By studying core oligosaccharides we observed that rhamnose and glucose were the main neutral sugars. All strains revealed KDO and αalanine.

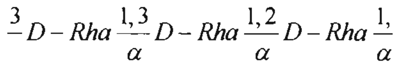
Inspite of the fact that the core is traditionally considered as more conservative part of the LPS molecule we have discovered three chemotypes of core oligosaccharides among II serogroup strains.

A study of the chemical composition of lipids A and core oligosaccharides did not shown any correlation between their composition and pathovar differentiation by host-plant.

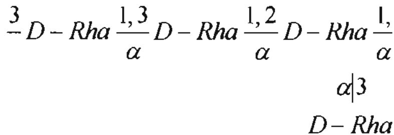
Since in literature there is information that the O-chain (O-specific polysaccharide) is responsible for serogrouping by thermostable antigens we have fulfilled the deep study of this part of the macromolecule LPS.

The deep structural analysis of O-PS by using ^{13}C-NMR-spectroscopy allowed to establish the structure of the repeating units of O-chain. The strains revealed one chemotype of O-PS but three types of their structure :

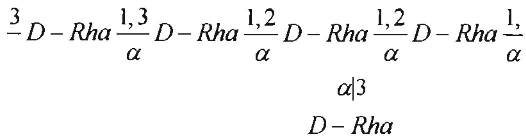
pv. *morsprunorum* CF-4, pv. *syringae* 467 :



pv. *syringae* 435 :



pv. *atrofaciens* K-1025, pv. *syringae* 90a :



Due to established heterogeneity of core composition, lipid A and O-PS within one serogroup, a detailed study of serological characteristics of the intact LPS macro-molecule as well as core, lipid A and O-PS has been made (Table 2).

Table 2 : Serological characteristics of the preparations

Pathovar, strain	Preparation	Titres (1) in reaction of ring precipitation with serum		The number of lines DDA with serum		The number of lines IEP with serum	
		OH-	O-	OH-	O-	OH-	O-
pv. *morsprunorum* CF-4	LPS	1000000	250000	3	2	2	2
	O-PS	500000	250000	1	1	1	1
	core	1000	1000	1	1		
	Lipid A	100000	100000	1	1	1	1
pv. *syringae* 90a	LPS	500000	500000	2	2	2	2
	O-PS	50000	50000	1	1	1	1
	core	1000	1000	1	1	1	1
	lipid A	100000	100000	1	1	1	1
pv. *syringae* 435	LPS	1000000	500000	2	2-3	2	2
	O-PS	100000	100000	1	1	1	1
	core	5000	5000	1	1		
	lipid A	500000	500000	1	1	1	1
pv. *syringae* 467	LPS	1000000	1000000	1	1	1	1
	O-PS	500000	500000	1	1	1	1
pv. *atrofaciens* K-1025	LPS	1000000	500000	2	2	2	2
	O-PS	100000	100000	1	1	1	1
	core	10000	10000	1	1		
	lipid A	500000	500000	1	1	1	1

All preparations we have examined had serological O-activity. In all cases the following regularity was observed : serologically high active LPS and lipid A, active O-PS and weakly active core. All preparations were active in reaction of double diffusion in agar (DDA) and immunoelectrophoresis (IEP). LPS preparations of all strains were active in a reaction of passive haemagglutination (RPHA). They had high inhibitory activity towards RPHA in a system of homologous strain since in heterologous system it was considerably lower (Table 3). The analogous results have been achieved at the inhibition of the system by O-PS, core and lipid A. In all cases the separate parts of LPS macromolecule were active in homologous systems and characterized by considerably lower or lack inhibitory activity in the systems heterologous strains. Strains 90a and K-1025, CF-4 and 467, having identical structures of O-PS, conduct themselves differently in the inhibition of RPHA (Table 3).

Table 3 :

The inhibition of RPHA by the preparations from serogroup II strains.

Strain system (O-serum + LPS)	RPHA (titre)	Inhibition of RPHA				
		strain		preparations (mg/ml)		
		inhibitor	LPS	O-PS	core	lipid A
pv. *morsprunorum*	5120	CF-4	8	1	32	32
CF-4		90a	500	-	-	-
		435	256	64	500	500
		467				
		K-1025	64	64	-	256
pv. *syringae*	2560	CF-4	256	32	-	-
435		90a	32	-	-	-
		435	0,25	4	64	64
		467				
		K-1025	128	256	128	256

High serological activity of lipid A makes it possible to suppose its important role in serogrouping of *P. syringae* strains.

As we study LPS bacteria pathogenic for plants we tried to elucidate the role the macromolecule and its components play in the infectious process. With this purpose the preparations of LPS, O-PS, core and lipid A were injected under epidermic cells of tobacco (leaves), tomatoes (fruits), bean (pods), Sudan grass (stems) and cabbage (leaves). The plants treated by cells of bacteria pathogens served as control. As a result, all bacterium-infected plants revealed symptoms characteristics for bacterial infection. As for plants response to LPS preparations, they gave some characteristic symptoms. These are spots (necrotic lesions) on stems of Sudan grass, water-soaked spots on tomatoes, chlorosis on tobacco leaves, weak tissue hyperplasia on cabbage leaves. However, after the injection into the plant of the preparations of some structural LPS components (core, O-PS and lipid A) we could not manage to vizualize symptoms of plant injury.

Thus, strains of serougroup II were shown to be heterogeneous by chemical composition of core, lipid A and structure of O-specific polysaccharide. Their heterogeneity in cross serological reactions has been demonstrated. O-specific polysaccharides having identical structures are not identical in cross serological reactions. High serological activity of lipid A makes it possible to suppose their important role in strain serogrouping. We confirmed that only the intact LPS preparations can induce symptoms characteristic for infection.

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Effect of *Pseudomonas syringae* pv. *phaseolicola* lipopolysaccharides on suspension cultured cells of *Lycopersicon peruvianum* Mill.

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Abstract

Lipopolysaccharides of *Pseudomonas syringae* pv. *phaseolicola*, the causal agent of halo blight of bean, were applied to suspension cultured plant cells of *Lycopersicon peruvianum* MILL. The parameters studied were growth, triphenyltetrazoliumchloride activity (TTC test for reductases), activities of intracellular peroxidase, polyphenol oxidase and phenylalanine ammonia-lyase and formation of ethylene. A significant increase of ethylene formation was detectable as early as 100 min after application of LPS. All measured enzyme activities increased as well, whereas the growth of plant cells was inhibited during the incubation time. The responses of the plant cells suggest a participation of LPS in resistance mechanisms like HR in incompatible plant-microbe interactions.

Keywords

Pseudomonas syringae pv. *phaseolicola*, *Lycopersicon peruvianum*, ethylene, lipopolysaccharides, cell suspension culture, hypersensitive response, peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase.

Introduction

Bacterial lipopolysaccharides (LPS) are known to serve as important factors for pathogenicity in bacteria-plant interactions for a long time. In view of their highly specific structure an participation of LPS in development of the strong host specificity of phytopathogenic pseudomonads is discussed by many authors. On the other hand LPS seem to induce phenomena of pathogenicity not only in compatible but also in incompatible interactions leading to resistance or

hypersensitive response (HR) of the invaded plant. The exact mechanism of the recognition processes probably proceeding during the first invasion steps is poorly understood at present. Suspension cultured plant cells are suitable systems for elucidation of the modes of action of pathogens or substances produced by them and plants or plant cells. The enlarged cell surface compared to intact plant tissue facilitates the specific contact with compounds involved in pathogenesis. Furthermore, monitoring of answers of the plant cells is much easier to practise and characterized by an enhanced reproducibility as compare to whole plant tissues. The purpose of this study was to find out the effects of purified LPS from *Pseudomonas syringae* pv. *phaseolicola* to plant cell cultures of *Lycopersicon peruvianum*. *P. s.* pv. *phaseolicola*, the incitant of halo blight of bush bean, causes HR when inoculated into whole *L. peruvianum* plants.

Material and methods

Cultivation of plant cells from *L. peruvianum*, estimation of dry mass, viability of cells and assays for polyphenol oxidase (PPO), peroxidase (PerO) and phenylalanine ammonia-lyase (PAL) are described by PERNER & SCHMAUDER (1992). The detection of ethylene followed the instruction of GREULICH *et al.* (1992).

LPS were isolated from *P. s.* pv. *phaseolicola* by phenol/water-extraction according to WESTPHAL *et al.* (1952) and further purified by enzymatic digestion of contaminants with α-amylase and RNase. After a second phenol/water-extraction LPS containing solution was dialysed and lyophilized. Before application to plant cell cultures, LPS were solubilised by sonication and sterilized by passage through a membrane filter (0.2 µm, SARTORIUS).

Results and discussion

Effect of LPS on dry mass

The first measurable inhibiting effect of LPS to plant cell cultures were detectable six days after inoculation. The cells´ response increased from 10 µg/ml and 50 µg/ml LPS, but did not differ if concentrations above 50 µg/ml LPS were used. LPS influenced the growth of plant cells only at relatively high concentrations compared with the phytotoxin coronatine wich affects cells already at 0.7 ng/ml. Furthermore, plant cells were not injured lethally and the growth was inhibited to a limited extent only (Fig. 1).

Effect of LPS on viability of plant cells

The reduction of TTC by extracts of plant cells was used as an indication of cell-viability. After application of LPS the activity of reductases increased strongly. The low concentrated LPS (10 µg/ml) evoked a weak reaction again, whereas after treatment with 50 µg/ml and 100 µg/ml LPS, respectively, the cells showed the same responses. Probably, the surviving cells of *L. peruvianum* activated their metabolism in order to compensate the growth-inhibiting action of LPS (Fig. 2).
For comparison: The phytotoxin coronatine lowered the TTC-activity (PERNER & SCHMAUDER 1993) although *L. peruvianum* is partially resistant against the producer of coronatine, *Pseudomonas syringae* pv. *tomato*.

Effect of LPS on enzyme activities

The intracellular activities of PAL, PerO and PPO increased already 12 hours after application of 100 µg/ml LPS. 10 µg/ml produced no significant effects (Fig. 3-5). As reviewed by WARD (1986) these enzymes may have an important function in production of antimicrobial compounds directed against the pathogens. For this reason, LPS might be possible signal molecules with a specific binding site to plant cell walls. From this interaction a cascade of reactions may result leading to the phenomena of resistance or HR. Various authors (see WARD, 1986) described an induction of the enzymes studied in our experiments by the phytohormone ethylene. Therefore we examined the influence of LPS application on ethylene production by plant cell cultures.

Effect of LPS on ethylene production

The induction of ethylene formation was detectable already 100 minutes after application of LPS and reached approximately 560% compared to the control after 6 hours. Also for 10 µg/ml LPS a significant response was detectable by GC (Fig. 6). We assume that the ethylene forming enzymes may be one of the first steps of the above mentioned cascade wich is switched on by an attachment of LPS or related signal molecules to the cell surface.
A rapid ethylene response of plants after invasion of incompatible pathogens may contribute to the very fast development of HR by inducing enzyme activities. The mode of action of enzyme induction and particularly the molecular basis of limited cell death need further investigations. Suspension cultured plant cells could be an important tool for this purpose.

Figures 1-6:

Effect of LPS from *P. s.* pv. *phaseolicola* on some physiological parameters of cell cultures of *Lycopersicon peruvianum.*

The sterile filtered solutions of LPS were given at the beginning of freshly inoculated plant cell cultures.

The following concentrations were used: ☐ control, addition of the same volume water instead of LPS, ▤ 10µg/ml LPS, ▥ 50µg/ml LPS, ■ 100µg/ml LPS

The following physiological markers were examined: Growth (Fig. 1), TTC activity (Fig. 2), PAL (Fig. 3), PerO (Fig. 4), PPO (Fig. 5), ethylene formation (Fig. 6)

Fig. 1: **Growth (dry mass)**

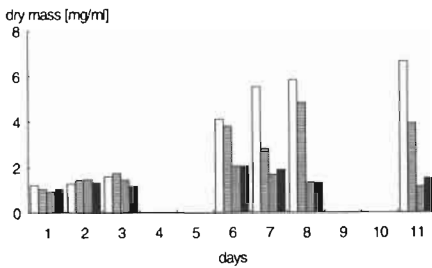


Fig. 2: **TTC activity (viability)**

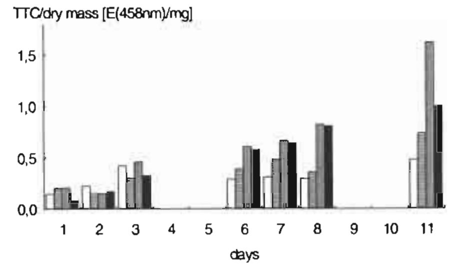


Fig. 3: **PAL**

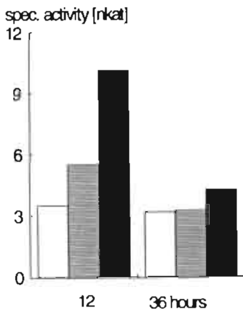


Fig. 4: **PerO**

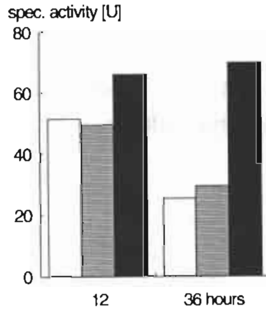


Fig. 5: **PPO**

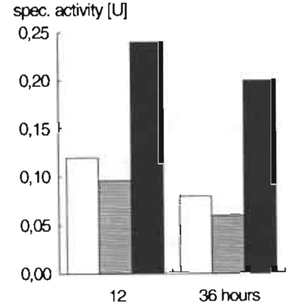
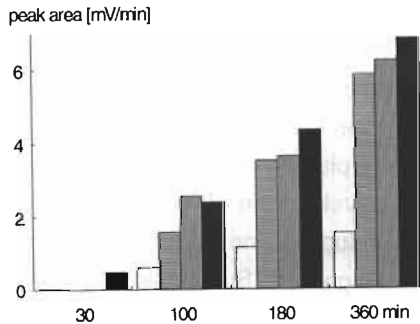


Fig. 6: **ethylene formation**



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The evaluation of tomato cultivars resistance to tomato wilt

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ABSTRACT

In the tomato plants of cv. Sláva Porýni, Bulgaria 12, Tornádo, Start F_1, Moneymaker and Imun resistance to *Clavibacter michiganensis* subsp. *michiganensis* was evaluated by means of conventional and biochemical methods. Results from both conventional and biochemical methods were compared. On the bases of results cv. Bulgaria 12 and Sláva Porýní were determined as resistant, cv. Start F_1, Moneymaker, Tornado and Imun as susceptible to bacterial wilt.

KEYWORDS

Tomato cultivars, *Clavibacter michiganensis* subsp. *michiganensis*, resistance, conventional and biochemical methods.

INTRODUCTION

In the last three years in Czechoslovakia a considerable increase of infection of tomato cultivars with *Clavibacter michiganensis* subsp. *michiganensis* was described. Earlier this disease was not significant in comparison with other areas of the world. The symptoms of the disease were described by many authors (STRIDER, 1969). The control methods were studied and applied, but in many cases they were unsuccessful. Therefore, studies were undertaken to investigate the perspectives of breeding for resistance in the USA, France, Japan and particularly in the Netherlands (STEEKELENBURG, 1985 ; GRIESBACH & KLEINHAUS, 1991).

The presented paper summarizes results of resistance evaluation of young tomato plants by means of conventional (STEEKELENBURG, 1985 ; KRATKA & KUDELA, 1987) and biochemical methods (KRATKA & KUDELA, 1987 ; KRATKA, 1990). Results from both conventional and biochemical methods were compared and their application was estimated.

MATERIALS AND METHODS

We studied 6 tomato cultivars : Bulgaria 12, Sláva Porýni, Imun, Tornádo, Start F_1 and Moneymaker. Tomato plants were grown in topsoil substrate in the greenhouse. The inoculation of plants with *Clavibacter michiganensis* subsp. *michiganensis* (CMM) was carried out at the three leaf stage. The roots were plunged into suspensions of bacteria (10^6) for the period of 18hrs. Both inoculated and control plants were sampled :

- 28 days after inoculation to analyse with conventional methods,
- 10 days after inoculation to analyse with biochemical methods.

The conventional method was based on the evaluation of leaves according to the disease symptoms. Each variant represented the mean of three replications (3 x 15 plants). We used the following scale for determination of degree of severity : 0 - healthy plant ; 1 - 1/4 of leaves wilt ; 2 - 1/3 ; 3 - 1/2 ; 4 - 2/3 ; 5 - all leaves wilt ; 6 - plant is extinct.

$$DS = \frac{n0.0 + n1.1 + \ldots\ldots n6.6}{N}$$

N = all plants

n = diseased plants

The biochemical method was based on the determination of changes of hydroxyproline (Hyp) content bound in glycoproteins of plant cell walls. The plants populations were evaluated as resistant according to the biochemical method when the content of Hyp after inoculation did not differ significantly from the control (uninoculated plants). On the contrary, the plants of susceptible populations accumulated a considerable amount of Hyp in the cell wall after inoculation (higher than 25%) (KRATKA, 1990).

The cell wall was obtained from roots (MASUDA *et al.*, 1982). Hyp content was determined spectrophotometrically (KIVIRIKKO *et al.*, 1965). Data are expressed as ug Hyp.mg^{-1} cell wall. Each variant represented the mean of three replications (3 x 15 plants).

RESULTS

On the bases of our results obtained by the conventional method we determined young plants of cv. Bulgaria 12 and Sláva Porýni as resistant (DS < 1,5), cv. Start F_1, Moneymaker, Tornado and Imun as susceptible (DS > 1,5) to bacterial wilt (Fig. 1).

Fig. 1 : Disease symptoms 28 days after inoculation

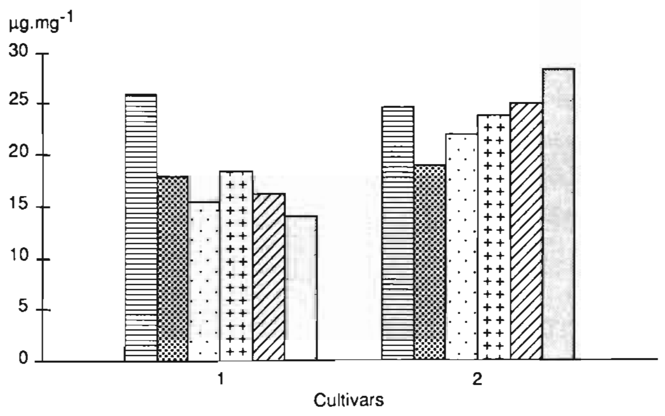

Fig 2 : Hydroxy proline (Hyp) content of the glycoproteins from root cell walls

Slava Poryni, Bulgaria 12, Tornado
Start F1, Moneymaker, Imun

1 - Control
2 - Infection

The results obtained by the biochemical method were similar (Fig. 2). The increase of Hyp content over 25% (in comparison with healthy plants) determined the wilt susceptible population. The Hyp content lower than 20-25% determined the wilt resistant population.

DISCUSSION

For determination of resistance of tomato genotypes to CMM many techniques of inoculation and evaluation of degree of severity were divised (STEEKELENBURG, 1985).

For our requirements the inoculation through roots was the best one.

A comparison of the changes of Hyp content in control and inoculated tomato plants with CMM shows that the results are in correlation with our recent data from other systems of plant - wilt pathogen. Our earlier results indicated that an increase lower than 20-25% of Hyp content in cell wall of diseased plants determined the wilt resistant population. The increase of Hyp content over 25-30% determined the wilt susceptible population (KRATKA, 1990).

Similar results were obtained by means of conventional or biochemical methods in the system tomato - CMM.

Considering the increase of tomato bacterial wilt in our country during the last years breeding of resistant cultivars is relevant. We suggest to screen breeding materials after suitable inoculation through the roots and biochemical evaluation of resistance the level. This method can be used 10 days after inoculation of young plants. At this wilt of leaves and discoloration of infected roots is just beginning and the severity of the disease can not be exactly determined. The biochemical method is fast, reproducible and reliable.

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Effects of inoculation of *Pseudomonas syringae* subsp. *savastonoi* on leaves and explants of oleander

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INTRODUCTION

Pseudomonas syringae subsp. *savastanoi* is a pathogen on olive and oleander. When inoculated, it induces knots by releasing indoleacetic acid (IAA) and cytokinins into the plant tissue at the site of inoculation (SMIDT & KOSUGE, 1978; SURICO *et al.*, 1985).
Strains from oleander knots produce symtoms on olive as well as on oleander, but strains from olive knots are avirulent on oleander (SUTIC & DOWSON, 1963; JANSE, 1981), and this even though they are not apparently different from the oleander strains in IAA and cytokinin production (SURICO & IACOBELLIS, 1992).
Despite extensive research on the pathogenicity of *P.s.*subsp. *savastanoi* strains, the reason for this resistance of oleander to the olive strain is still unclear. The present study examines some aspects of knot formation by *P.s.* subsp. *savastanoi* using inoculation on oleander leaves, explants, and cultured oleander cells in suspension.

MATERIALS AND METHODS

Inoculation tests

In vivo inoculations. The knot-inducing capacity of the selected strains was tested on leaves of 2-year-old seedlings of *Nerium oleander*. Suspensions of approximately 10^7, 10^8 or 10^9 cfu/ml sterile distilled water (SDW) were infiltrated into the underside of veinous and interveinous tissue using a 26-gauge hypodermic syringe.
Explant inoculation. Pieces of mature leaves of N. *oleander* were cut out with a scalpel, sterilized and transferred to basic medium of Murashige and Skoog supplemented with only 3% sucrose (MS-1). Inoculation was performed by depositing 5 µl of a $2X10^8$ cfu/ml bacterial suspension on an exposed vein. Control explants were inoculated with SDW. To test for callus production, other leaf pieces were placed on MS-1 that had been further supplemented with 0.1 mg/l BAP and 1 mg/l 2,4 D (MS-2).

Tab. 1 - Strains of *Pseudomonas syringae* subsp. *savastanoi* used in this study.

Strain	Host origin	Phytohormone phenotype
ITM519	Oleander	IAA^+/cytokinins$^+$
ITM519-41	Oleander	IAA^+/cytokinins$^-$
ITM519-7	Oleander	IAA^-/cytokinins$^+$
ITM519-6	Oleander	IAA^-/cytokinins$^-$
ITM317	Olive	IAA^+/cytokinins$^+$

IAA = indole-3-acetic acid

Bacterial multiplication

Leaves. At days 0,1,3,5,7,9,11 and 15 after inoculation, portions of inoculated tissue were rinsed with SDW, wiped, weighed and homogenized in a mortar with SDW. The resulting suspensions were serially diluted and plated on King's B agar plates for colony counts.

Explants. Explants inoculated with ITM519, ITM519-6 ITM519-7, ITM519-41 and ITM317 were sampled before inoculation and at 1,3,5,10,17,25 and 45 days after inoculation. Three explants per strain were weighed and ground in a sterile mortar containing 1 ml SDW. Decimal serial dilutions were plated on King's B agar plates. The number of bacteria per g leaf tissue was calculated from colony counts after 3 days at 26°C.

Bacterial attachment to oleander cells

Oleander cell suspension cultures were started in MS-2 broth from 30-day-old callus tissue. Cultures were inoculated with either *P.s.* subsp. *savastanoi* strain ITM317 or strain ITM519. To assess bacterial attachment, the inoculum of the strains was adjusted to give a ratio of bacteria to plant cells of about 30:1.

At 0.5,2,4,6 and 24 h after inoculation, 2-ml aliquots from the inoculated cell cultures were taken and filtered through a Millipore SC membrane (8 µm pore size) that retained oleander cells and any bacteria attached to them but allowed free bacteria to pass through. The filter was then washed in 5 ml SDW and the filter washing waters, the filtrates and 0.1 ml samples of inoculated oleander cells were plated to determine concentrations of viable bacteria. The number of free plus attached bacteria equalled the total number of bacteria in the oleander cell suspension culture.

Oleander cell viability was determined throughout a sampling period of 7 days by vital coloration with 0.5% fluorescein diacetate in acetone.

Scanning electron microscopy

Oleander cells on the Millipore filters were fixed in 2.5% glutaraldehyde postfixed in 1% osmium tetroxide, and dehydrated in successive ethanol solutions. Specimens were dried in a critical point drier under CO_2, mounted on specimen holders, coated with gold-palladium, and viewed in a Philips SEM505 scanning electron microscope.

RESULTS

Inoculation tests

In vivo inoculation. *P.s.* subsp. *savastanoi* oleander strain ITM519 was pathogenic towards oleander plants at all concentrations (10^7, 10^8 and 10^9 cfu/ml), producing knots at the site of inoculation within 15 days.

The wild-type olive strain and the IAA^- mutants of the oleander strain induced only necrotic, and sometimes chlorotic symptoms within 24-48 h and only at 10^8 and 10^9 cfu/ml. At 10^7 cfu/ml these strains did not induce any symtoms. The mutant ITM519-41 (IAA^+/citokinin$^-$) induced small knots at 10^9 cfu/ml.

Explant inoculation. All the strains inoculated on leaf explants induced tissue proliferation except the IAA^- mutant strains. The wild-type oleander strain ITM519 induced proliferation of white to light-green tissue after 7-10 days, and the olive strain and ITM 519-41 (IAA^+/cytokinins$^-$) also induced proliferation, though not until 15-20 days after inoculation.

The IAA^- mutant strains did not lead to tissue proliferation, but they heavily colonised the explants, which eventually became partly or totally necrotic. On MS2 uninoculated explants produced abundant tissue proliferation.

Bacterial multiplication

Leaves. The ITM519 oleander strain and its mutants which were defective in phytohormone production all multiplied in oleander leaves at a similar rate and reached a similar population size.

The ITM317 olive strain multiplied less well than the oleander strains and had a lower population size by the end of the experiment.

Explants. Among the interactions tested the two wild type strains ITM317 and ITM519 multiplied steadely to reach about $3x10^9$ cfu per g leaf tissue by the end of the experiment. By contrast the mutats without phytohormone production began to decline in numbers after 25 days.

Cell suspension cultures. Multiplication of ITM519 was not stimulated significantly by the presence of the oleander
cells in MS-2 culture medium. By contrast, ITM317 had a final population size after 24 h that was about 1.5 times higher in the presence of oleander cells thant without. Oleander cells viability was slightly reduced by both strains during the first 24 h and much more markedly thereaflter.

Bacterial attachment to oleander cells

Both the ITM519 and the ITM317 strains became attached to cultured oleander cells.
At 6 and 24 h from inoculation over 40% of both strains appeared associated with oleander cells after fractionation. Examination by scanning electron microscopy corroborated the findings of the binding experiments.

CONCLUSIONS

The inoculation tests on the leaves confirmed the avirulence of the olive strains of *P.s.* subsp. *savastanoi* on oleander. Strains ITM317 multiplied in oleander leaves but did not induce knots.
When inoculated on the leaf explants, however, ITM317 induced abundant tissue proliferation. This shows that the bacteria had not lost their ability to synthetize IAA - and hence to induce hyperplasia. Clearly knot formation in oleander inoculated with the olive strain was blocked at some later stage than IAA synthesis.
The primary role of IAA in knot induction was confirmed. Of the mutants tested only ITM519-41, the mutant with IAA production (IAA^+/$cytokinins^-$), multiplied in both oleander leaves and explants and also induced small knots on the leaves and abundant proliferation in the explants. Numerous roots later developed from the resulting calli, indicating a high IAA to cytokinin ratio.
Inoculation of ITM317 and ITM519 on cultured oleander cells in MS-2 medium revealed only minor differences between ITM317 and ITM519. Both strains - especially ITM317 - multiplied slightly better with the oleander cell suspension cultures than without. This may indicate that oleander cells release constituents that the bacteria feed upon.
Oleander cell viability was not significantly reduced 24 h after ITM317 or ITM519 was added to the suspension culture. On oleander leaves inoculated *in vivo* with these strains, viability was also unaffected except at high doses (10^8 and 10^9 cfu/ml for ITM317 and 10^9 for ITM519), when rapid necrosis was noted.This necrosis may therefore be attributed to the high dosages.

Examination by scanning electron microscopy revealed that ITM317 and ITM519 cells became attached to oleander cells. Since ITM519 caused disease and ITM317 did not, the fact of bacterial attachment does not therefore explain virulence.
Attachment of course remains a possible factor in the pathogenicity of *P.s.* subsp. *savastanoi*.

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Possible functions for *Pseudomonas solanacearum* *hrp* genes and conservation among Gram negative phytopathogenic bacteria

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ABSTRACT

Genetic approach of pathogenicity determinants in *Pseudomonas solanacearum* strain GMI1000 had allowed the identification of *hrp* genes which are essential in governing the ability of the bacteria to cause disease on host tomato and the ability to induce a Hypersensitive Response on tobacco. These genes have been shown to map in a 23 kb gene cluster and to be organized in a minimum of six transcriptional units.

Sequencing of 20 kb of DNA located at the left end of this cluster has revealed a minimum pf 19 Open Reading Frames (ORFs) with a high coding probability. Analysis of the amino acid sequences of the corresponding putative poplypeptides revealed that:

-At least 7 of these polypeptides have clear characteristics of membrane bound proteins,

-That one ORF codes for a protein homologous to the alpha chain of the CF1 component of bacterial ATP synthases,

-That 3 other ORFs (*hrpA, hrpl and hrpO*) code for proteins respectively homologous to the proteins LcrD, YscJ and YscC of the human and animal pathogen *Yersinia*. In this organism these proteins are components of a secretory machinery required for the secretion of the Yops proteins which are primary determinants of pathogenicity.

Altogether these data suggest that *hrp* genes could be involved in the secretion of macromolecular compound(s) required for the triggering of the plant response.

An additional ORF has been shown to code for a positive regulator required for the transcription of at least 4 of the 6 *hrp* transcription units previously identified. This regulator shows homology with other transcriptional activators of the *AraC* family including *virF* which regulates the transcription of the entire regulon coding for the Yops secretion.

Probes consisting of DNA fragment internal to *hrpA* and *hrpl* have been shown to hybridize with the hrp gene cluster from Xanthomonas campestris. Similar experiments using a hrpO DNA probe revealed the presence of homologous sequences within the hrp gene clusters from *X.campestris*, *P.syringae* and *Erwinia amylovora*. This is in favor of the existence of a common core of hrp genes among major representatives of the gram negative phytopathogenic bacteria.

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Cloning of an avirulence gene *avrPmaA2.Rpm1* from *Pseudomonas syringae* pv. *maculicola* which confers ecotype specificity in *Arabidopsis thaliana*

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ABSTRACT

An avirulence gene isolated from race 2 of *Pseudomonas syringae* pv. *pisi* (*Ppi*) and designated *avrPpiA.R2*, confers avirulence toward pea cultivars harbouring the resistance gene R2. Using DNA hybridization, this gene was used to probe a partial plasmid DNA library from *P. syringae* pv. *maculicola (Pma)* strain HRI791 to identify potentially homologous clones. One hybridizing clone, designated pAV500, comprised about 8.5kb of insert DNA in pLAFR3. Introduction of pAV500 into Ppi confers cultivar-specificity toward pea in a manner identical to that shown by *avrPpiA1*, indicating that the cloned *Pma* gene also behaves as a determinant toward non-host resistance in pea: thus R2 may function both in a race-specific and in a non-host resistance capacity. Isolates of Pma exhibit ecotype specificity toward accessions of *Arabidopsis thaliana*. Introduction of pAV500 into Pma strain m4, which is compatible on a number of ecotypes, confers ecotype-specific avirulence. Further analysis of pAV500 has revealed that a region of about 2kb is responsible for the cross-hybridization observed with *avrPpiA1*, and that both share homology with a further gene, *avrRpm1*, cloned independently by screening a library from *Pma* strain m2 introduced into strain m4. All three avirulence genes are apparently similar if not identical, and progress with the elucidation of their relationship to one another will be reported.

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Production of syringotoxin by *Pseudomonas fuscovaginae*

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ABSTRACT

Strain UPB 264 of *Pseudomonas fuscovaginae* has been shown to excrete *in vitro* phytotoxic peptides, reproducing leaf sheath necrosis and inhibition of panicle emergence, typical of bacterial sheat rot of rice. After extraction from the liquid culture of P. *fuscovaginae* and purification by reverse phase HPLC, the most abundant toxic peptide was characterized by amino acid analysis, fatty acid methyl ester analysis and fast atom bombardment - mass spectrometry (FAB-MS). Equimolar ratio of threonine, serine, glycine, ornithinine and 2,4- diaminobutyric acid were identified in the elution pattern of the 6 N HC1-hydrolysate obtained from the purified peptide. Fatty acid methyl ester analysis of the HC1-hydrolysate revealed the presence of hydroxytetradecanoic acid. Using FAB-MS, the MH^+ion of the peptide was a doublet at the mass 1136-1138 which typically indicate the presence of a chlorine atom in the molecule. Analysis by FAB-MS of by-products from soft alkaline hydrolysis of the purified peptide shown a single MH^+ ion at 1136 and several other fragments at *m/z* 1018, 887, 870, 804, 787, 703, 686, 572, 488, 431, 414, 314 and 286. These results suggests that the toxic peptide is identical to syringotoxin ($C_{48}H_{82}O_{18}N_{11}C1$), a phytotoxin produced by *Pseudomonas syringae pv. syringae*.

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Biological properties of *Pseudomonas syringae* pv. *syringae* toxins, syringomycins and syringopeptins

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ABSTRACT

Most *Pseudomonas syringae* pv. *syringae* strains produce in culture syringomycin (SR), a toxin which has been reported to inhibit the growth of several microorganisms and to be implicated in the symptom expression of diseases caused by the bacterium. Actually, SR preparations, previously considered homogeneous, are complex mixtures of structurally related lipodepsipeptides, syringomycins (SRs) and syringopeptins (SPs).

In the antimicrobial assay on yeasts, fungi and bacteria, Srs were remarkably more active than Sps. In particular, SRE, the main component of SRs, was 30 times more active than SP_{25A} when assayed on *Rhodotorula pilimanae*, which is used with *Geotrichum candidum* to determine in the plate assay the SR produced by *P.s.* pv. *syringae* strains. By contrast, when the toxins were assayed for their phytotoxicity, determined as induction of electrolyte leakage from carrot tissues, necrosis of tobacco leaves and loss of the protoplast viability in potato tissues, SPs were more active than SRs. In particular, SP_{25A} was at least 40 times more active than SRE in inducing electrolyte leakage from carrot disc tissues. SP_{25A} was also much more active than SRE in inducing necrosis of tobacco and a rapid death of potato protoplasts. When assayed on human erythrocytes, SPs induced rapid cell lysis while no effect was observed for equimolar SRs solutions.

The above findings indicate that SRs and SPs are mainly responsible for, respectively, the antimicrobial and the phytotoxic activities characteristic of unfractionated SR. Furthermore, while the activity of SRs has recently been associated with biochemical effects at the level of plasma membrane, the high rapid activity of SPs on potato protoplasts and on human erythrocyte viability, and the induction of a high rapid level of electrolyte leakage from carrot tissues suggest that SPs may be responsible for the destructive action of SR on the membranes reported by DeVay and his colleagues.

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Study of *out* genes of *Erwinia chrysanthemi*

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ABSTRACT

Erwinia chrysanthemi 3937 selectively secretes six pectinases, one cellulase, and proteases into the outer medium.
To study the secretion of these enzymes, we have obtained secretion deficient mutants by insertion of transposable elements derived from the phage Mu (Mini Mu 1734) into the chromosome. These mutants, called Out$^-$, produce normal quantities of pectinases and cellulase, but do not secrete them. These enzymes accumulate in the periplasmic space. On the other hand, the Out$^-$ mutants normally secrete their proteases.
A gene library of E. chrysanthemi has been constructed, using cosmid, and screened with several cloned *out* mutations as probes. This yielded several cosmid clones carrying three different cluster. The loci were mapped on the chromosome of E. chrysanthemi. One of these cluster, named *out1*, was subcloned.
To identify the *out* genes present in this cluster, the nucleotide sequence of a 7393 bp*Eco*R1-*Bam*H1 fragment was determined on both strands. This fragment contains six open reading frames, plus the 3' end of a seventh one.
Sequence analysis of the six *out* genes (*out*I, *out*J, *out*K, *out*L, *out*M, *out*O) revealed that they are organized in a single operon. The size of the predicted products is 14 KD, 27.5 KD, 38.6 KD, 45,5 KD, 18,4 KD and 42.6 KD respectively.
Proteins corresponding to these predicted sizes were detected when the genes were expressed under T7 promoter control.
A comparative study of the *out* genes of *E. chrysanthemi* 3937 showed:
- A high homology with the end of the *pul* operon of *Klebsiella pneumoniae*,
- A different degree of homology with genes involved in protein secretion from some Gram-negative bacteria: *Xanthomonas campestris*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, etc...

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The pectin methyl esterase of *Erwinia chrysanthemi* strain 3937: sequence and overexpression

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ABSTRACT

The enterobacterium *Erwinia chrysanthemi* is pathogenic on many plants, monocots as well as dicots in fields or during storage. The most usual symptom observed is a soft rot on the infected leaf followed by a systemic invasion through the vascular system. This symptom is due to the production by the bacterium of a whole set of extracellular enzymes involved in plant cell wall degradation. *Erwinia chrysanthemi* strain 3937, synthesizes and secretes pectinases (5 pectate lyases and 1 pectin methylesterase), cellulases and proteases. The pectinases and the cellulases, representing five groups by their peptidic sequence, are secreted by the same system (*out*), different from the one of proteases.

Among the pectinases, the pectin methyl esterase (PME) has been shown to be necessary to the systemic spread of the bacterium. Indeed, a mutant that does not synthesize this enzyme develops maceration only at the infection site. Moreover, PME, wich demethylates the pectin, allows further activity of the PLs (depolymerisation of the pectin) which are susceptible to the methylation degree of the substract. This leads to suppose that PME plays an essential role, being one of the first enzymes to act after a putative recognition step beetween the host and the pathogen.

The gene encoding the PME (pem) was cloned as a 4.6 kbp fragment. It was then subcloned as a 1.8 kbp fragment and sequenced by the "Shotgun" method. The sequence of *pem* showed an open reading frame 1098 bp in length. Upstream of the gene, can be observed (i) a Shine-Dalgarno sequence at 7 bases from the ATG codon, (ii) a region presenting 40% homology with the synthetic consensus promotor of *Escherichia coli*, at a 40 bases distance, and (iii) on the same locus, *a kdgrR* site. This site, highly conserved, is present upstream all the genes involved in the catabolism of pectin of *Erwinia chrysanthemi*. It probably corresponds to the recognition site for a negative regulator, translated from the *kdgR* gene. Downstream the pem gene, two terminator sites overlap,

one being *p*-dependent and the other *p*-independent. The *pem* ORF is translated into a 366 amino acids protein. Its NH_2-terminal sequence presents all the characteristics of a signal peptide with a cleavage site supposed to be between Ala_{24} and Ala_{25}. The mature protein has a molecular weight of 36.95 kDa and a calculated isoelectric point of 9.64.

PME, as the other pectinolytic enzymes of Erwinia chrysanthemi, is characterized by a high specific activity, but a low production. Therefore, it is necessary to overproduce the enzyme by genetic engineering before any attempt of purification and biochemical or biophysical study. A fragment carrying the *pem* gene and its transcription stops was amplified by PCR. It was cloned in the plasmid pPLc321 carrying the pL promoter of the λ phage and a Shine-Dalgarno sequence. The resulting plasmid was named pPLcpem. A DNA fragment carrying the pL promoter and the *pem* gene was amplified from pPLcpem, using the same method, and cloned in the high copy number plasmid pUBS3. The resulting plasmid was named pUBSpLpem.

Several strains of *Escherichia coli* (DH5α, C600) and *Erwinia chrysanthemi* (3937, 4072 Peld$^-$,e$^-$) were transformed by electroporation with these plasmids. In C600 pUBSpLpem, the PME activity was increased more than 30 fold, compared to the wild type strain 3937. Surprisingly, half of this activity is found in the supernatant. Analysis of the supernatant and the periplasm of this strain by electrofocusing and SDS-PAGE electrophoresis showed that the protein produced is, in all cases, the mature form.

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Isolation and characterization of promoters under positive regulation from *Xanthomonas campestris* pv. *campestris*

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ABSTRACT

The interaction of a plant pathogen with its host plant is a dynamic process which involves signal exchange between cells. *Xanthomonas campestris* pv. *campestris*, a pathogen of cruciferous plants, possesses a positive regulatory system containing a gene, *rpfC*, the product of which has homology to conserved regions of both sensor and regulator proteins of procaryotic two-component regulatory systems (Tang et al., 1991), which are usually involved in regulating gene expression in response to environmental stimuli. The aim of the work is to isolate positively regulated promoters and their corresponding genes from *X.c.* pv. *campestris*. We screened a *X.c.* pv.*campestris* genomic library of 8000 clones constructed in a promoter-probe plasmid pIJ3100 (Osbourn et al., 1987), containing a promoterless chloramphenicol acetyl transferase gene, for promoters under positive regulation. Sixty clones contained a putative promoter fragment. The promoters were screened by transferring into a non-pathogenic mutant defective in positive regulation. Five clones showing reduced resistance to chloramphenicol in the mutant strain were subcloned into pUC19 vector for restriction analysis and sequencing. These sequences will be compared to those independent of positive regulation and consensus sequences are searched for.
The five promoter-carrying plasmids were also transferred into various bacterial strains including *X.c.* pv. *translucens*, *X.c.* pv. *vesicatoria*, *Pseudomonas syringae*, P. *putida*, *Klebsiella pneumoniae*, and *E. coli*. The function of the promoters correlated approximately with the systematic distance between the bacteria. One promoter was functional in all strains whereas most were functional only in *Xanthomonas* and *Pseudomonas syringae*.

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DNA sequence analysis of the avirulence gene *avrPpiA1.R2* from *Pseudomonas syringae* pv. *pisi* and its relationship to similar genes cloned from *P. syringae* pv. *maculicola*

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ABSTRACT

Pseudomonas syringae pv. *pisi* (*Ppi*) is the cause of bacterial blight of pea. Seven races of the pathogen have been distinguished by inoculation into a series of 8 differential cultivars of pea (*Pisum sativum*). The pattern of interaction can be explained in terms of five matching gene pairs for avirulence and resistance in the pathogen and host, respectively. A single avirulence gene, *avrPpiA1* (previously designated *avrAspi1*; Vivian *et al.*, 1989), cloned from race 2 was identified by its ability to confer avirulence in race 1 toward pea cultivars harbouring resistance gene R2. Hence the full current designation, to reflect the interactive nature of *avr* genes, is *avrPpiA1.R2*. The introduction of this gene into a wider range of races and their interaction with the full set of pea differentials will be reported. These results confirm the cultivar-specific nature of this gene. The location of *avrPpiA1* on the subclone pAV200 (4.1kb) was achieved through further subcloning and transposon mutagenesis using Tn*5* and Tn*3*HoKmGus. Both strands of a region of DNA encompassing the transposon insertions that abolished avirulence activity have been sequenced. This has revealed a single ORF of 660bp potentially coding for a protein product of about 28kDa. Analysis of the sequence has shown that the putative protein is hydrophilic in nature and devoid of known signal sequences, suggesting a cytosomal location in the bacterium. Searches using SEQNET at Daresbury of the GenBank and EMBL databases have failed to reveal significant homology to any known protein. Two potential homologues of *avrPpiA1* have been isolated, namely *avrPmaA2.RPM1* (Vivian *et al.*, 1992) and *avrRpm1* (Debener *et al.*, 1991); both of these genes are being sequenced for comparison with *avrPpiA1*.

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Biochemical and molecular analysis of auxin production by various pathovars of *Pseudomonas syringae*

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ABSTRACT

Auxin (IAA) production by strains of *Pseudomonas syringae* pv. *savastanoi* was analyzed with the aim of looking for correlations among this characteristic and the origin of the strains, the types of symptoms, and the host plants. To do so, over 130 strains of *Pseudomonas* were isolated at different times, locations and from various plants.

In the first part of the study, auxin production was assessed in bacterial culture supernatants using a colorimetric technique (modified Salkowski's reagent). All strains of *Pseudomonas syringae* pv. *savastanoi* isolated from jasmine, *Phillyrea*, forsythia, privet, olive and oleander produced auxin. However, out of 33 strains isolated form ash, only 2 were found to produce IAA. Using a colony hybridization technique and a DNA probe encompassing genes *iaa*M and *iaa*H involved in auxin production, we observed that each and every strain producing auxin hybridized with the probe. It is noteworthy that symptoms of the disease on ash are different from those observed on olive and oleander. This feature, however, cannot be attributed to the single lack of IAA production, since the symtoms of the disease induced by the two atypical isolates were identical to those induced by other ash strains. Interestingly, the two ash strains producing IAA and harboring *iaa* genes were isolated in Algeria, a country were ash was only recently introduced. However, cross inoculations performed on various test-plants demonstrated that the two atypical Algerian isolates are *bona fide* ash strains and not olive or oleander strains which became virulent on ash. The origin of the *iaa* genes in these strains was investigated but remained unclear. Since auxin is not absolutely required for disease development on ash, presence of these genes could improve the epiphytic survival of these strains.

In the second part of our study, we analyzed auxin production by other pathovars of *Pseudomonas syringae*. Salkowski-positive compounds were thus detected in culture supernatants of P. *syringae* pv. *syringae*, pv. *tomato*, and pv. p*haseolicola*. However, colony hybridization did not revealed presence of *iaa* genes. Wether this is due to presence of more distanly-related *iaa* genes in these strains, and wheter the Salkowski positive compound is indeed auxin remain unknown.

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A comparative study of some plant pathogenic and plant deleterious pseudomonads

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ABSTRACT

The phytopathogenic bacteria *Pseudomonas syringae pv. pisi* and *P.s. pv. phaseolicola* cause bacterial blight and halo blight in peas and beans respectively. In a study on interactions between rhizosphere bacteria and plants, various strains identified as *P. fluorescens* were found deleterious to growth of different plant species in several greenhouse experiments. Comparison of some such strains (AT8, MA 250, and A112) with the pathogenic strains (*P.s. pisi* race1, 2 and *P.s. phaseolicola* race 1) revealed that none of the deleterious strains nucleated ice at temperatures above - 10°C while the *P. syringae* strains were able to nucleate ice between - 2°C and - 9°C. Assays on *in vitro* production of bacterial metabolites showed that the cell-free filtrates of all test strains cultured in nutrient broth possessed strong phytotoxic activity and that two of the deleterious strains were cyanogenic. In its natural host, *Phaseolus vulgaris*, the pathogen *P.s. phaseolicola* race1 introduced on seed or in plants causes halo blight. Seed inoculation of the same bean cultivar with either *P.s. pisi* or the deleterious strains also resulted in reduced seed emergence and retarded shoot growth in greenhouse. The hypersensitive response (HR) of tobacco leaves to hypodermic infiltration with the phytopathogens was confirmed in the nonhost broad bean (cv. Major) leaves. The existence of a typical HR response was also demonstrated in deleterious pseudomonad and broad bean conbination although the effective dose required for inducing HR was $>10^6$ cfu / ml. Recognition of HR induced within 6-24 hrs by both bacterial groups prompted further study on characterization of the response by *P. s. pisi* race 1 and AT 8 according to several criteria. A sharp decline in concentration of *P. s. pisi* race 1 *in vivo* was found after 4 h inoculation while a slight decrease followed by a slow increase in concentration was observed for AT 8. Although the electrolyte-loss from the broad bean leaves 3 h after inoculation with *P. s. pisi* race 1 was higher than that caused by AT 8, the induced loss and the change in pH during the first 6 h induced by AT 8 followed a similar pattern.

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Fimbriae in adhesion and pathogenicity of a potato pathogenic *Erwinia carotovora* subsp. *carotovora*

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ABSTRACT

Erwinia carotovora causes soft rots and other diseases in plants. *E. carotovora* subsp. *carotovora* produces mannose-binding type 1 fimbriae. In contrast, these fimbriae were not detected in other soft rot causing species *E. c.* subsp. *atroceptica*, *E. c.* subsp. *betavasculorum*, *E. chrysantemi* and *E. rhapontici*. The possible role of fimbria-mediated adhesion in pathogenesis of *E. c.* subsp. *carotovora*, was assessed in two experimental approaches: Pieces from potato tuber surfaces or potato leaves were incubated with bacteria, washed and examined by scanning electron microscopy. In the second approach, potato tubers were incubated with bacteria, and after washing the tubers were incubated and progress of soft rot symptoms was followed. Effect of α-methil-D-mannoside, a specific receptor analog of type-1-fimbrial binding, was also tested in both experiments. Scanning electron microscopy showed that *E. c.* subsp. *carotovora* adhered in high numbers to the surface of the potato tubers and leaves. The adhesion was inhibited by α-methyl-D-mannoside. Incubation with bacteria caused the symptoms of soft rot in potato tubers, the frequency of symptoms was lower in plants infected in the presence of α-methyl-D-mannoside. Our results indicate that *E. c.* subsp. *carotovora* has the capacity to adhere to potato tubers and that fimbriae are involved in this process. Adhesion to potato tubers increases the pathogenic potential of the bacteria by allowing colonization of plant surfaces and efficient access to sites of invasion.

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Enzymatic activities in relation to carbohydrate breakdown during pathogenesis of tomato fruits infected with *Xanthomonas campestris* pv. *vesicatoria*

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ABSTRACT

Concomitant analyses of the amounts of reducing non-reducing and total sugars and activities of α - amylase, invertase and α - glucosidase were carried out in the fruits of tomato cvs. 'Pusa Ruby' and 'Punjab Chhuhara' under the compelling influence of the bacterium *Xanthomonas campestris* pv. *vesicatoria* during 24 to 120h of pathogenesis. The investigations were carried out at 24 h interval. The enzymes α - amylase (3.2.1.1.: α -1, 4-glucan, 4-glucano hydrolase), invertase (3.2.1.26: β -D-fructofuranoside, fructohydrolase), and -glucosidase (3.2.1.20: α -D-glucoside, glucohydrolase) were closely concerned in affecting breakdown of the carbohydrates of the tomato fruits during the infection of the bacterium. They caused hydrolysis of starch sucrose and maltose leading to change in the balance of reducing and non-reducing sugars in the fruits of both the tomato cultivars. The action of these enzymes were at times synchronous and at times independant of each other. Glucose accumulated in 72 h and 96 h inoculated fruits due to the activity of the enzyme invertase. The released glucose units at other stages of pathogenesis were presumably not consumed by the bacteria and were used up for the synthesis of fresh non-reducing sugars.

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Growth kinetics and toxin production of *Pseudomonas syringae* pv. *glycinea* in planta

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ABSTRACT

Pseudomonas syringae pv. *glycinea* (Psg) causes bacterial blight of soybeans visible as foliar chlorosis in form of a halo around a necrotic lesion and can produce the phytotoxin coronatine. Out of 179 isolates obtained in 1983- 1990 from lesions of field-grown soybean plants one third failed to produce coronatine. The function of coronatine in pathogenesis was investigated. A bacterial suspension was applied to first fully expanded trifoliate leaves using tissue infiltration by pressure sprayer. The growth kinetics and the toxin production of Psg in leaves of host (soybean) and nonhost plants (bush bean) were monitored daily.
There were almost no differences in bacterial growth rates and final bacterial populations between the coronatine-producing and non-producing strains. The bacteria developed in the host and nonhost plants with similar growth rates. However, the final bacterial population of the heterologous combination was about one third of the population in the homologous combination. From 3rd day coronatine was detectable both in soybean leaves and in bean leaves. The coronatine concentration increased parallel to the bacterial growth. No evidence was found indicating that toxin production affects survival, bacterial multiplication or spread in planta. However there are differences in terms of symptom expression. The toxin-producer caused typical symptoms in host and nonhost plants. The non-producer developed only on the host plant disease symptoms.

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Chemical and biological features of cytokinins and auxins from phytopathogenic *Pseudomonas* species

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ABSTRACT

Zeatin (**Z**), 1'-methylzeatin (**1'MeZ**) and their 9-β-D-ribosides (**ZR** and **1"MeZR**, respectively) were isolated together with indol - 3 - aldehyde, indol- 3 - acetic acid (**IAA**) and some conjugates of **IAA** with *L*-lysine from the culture filtrate of *Pseudomonas syringae* pv. *savastanoi*, the causal agent of olive knot disease. These and other plant growth substances (PGS), namely **IAA**, **Z**, **IAA**-methyl ester, isopentenyladenine (iP), dihydrozeatin (**diHZ**) and 2'- deoxyzeatin riboside (**2' deOZR**) accumulated in cultures of *Pseudomonas amygdali*, the causal agent of hyperplastic bacterial canker of almond. Genetic analysis revealed a high homology between the DNA sequences involved in PGS synthesis by the two bacteria. The structure of **1' Mez**, **1"MeZR** and **2'deOZR** was confirmed by their total stereospecific synthesis, which also made it possible to obtain the unnatural *S*-diastereomers of **1'MeZ** and its 9-β-D-riboside and the *cis*-isomer of the **2' deOZR**. Moreover, the conjugates of **IAA** with *L*-lysine, namely **ε-IAA-Lys** and **Acetyl-ε -IAA-Lys**, and their unnatural α-analogs were obtained by a new stereospecific synthesis. Some of these synthetic compounds and other derivatives and analogs were evaluated in structure-activity relationship studies in comparing them with the natural metabolites. The length and the functionalization of the alkyl side chain as well as the integrity of the ribosyl moiety of the zeatin-like cytokinins were important in stimulating chlorophyll synthesis in etiolated cucumber cotyledons. The **ε-IAA-Lys** conjugates stimulated wheat coleoptile growth although the effect was much less than with **IAA**. By contrast the unnatural α-analogs were inactive. A FAB MS/MS method, suitable for a rapid and diagnostic analysis of cytokinins, will be also discussed.

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Influence of Ca^{2+} on pectate lyase production in *Pseudomonas marginalis*

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ABSTRACT

Pseudomonas marginalis is the causal organism of soft rot disease of vegetables in storage. It produces large amount of pectate lyase enzyme in medium supplemented with pectin. In *P. fluorescens* and *Erwinia chrysanthemi*, production and translocation of pectate lyase were greatly influenced by the divalant cation $(Ca)^{2+}$.

We studied the influence of Ca^{2+} on pectate lyase production in *P. marginalis*. Minimal medium (Na_2 HPO_2 -6 g, KH_2 PO_4 -3 g, NaCl - 0.5 g and $MgSO_4$ $7H_2$ 0 - 1 mM) containing different carbon sources like glucose, glycerol (0.2%), pectin, sodium polypectate (0.4%) and glucose+PGA (0.2+0.2%) were used. A concentration of 1 mM $CaCl_2$ was used in this study. The enzyme levels in medium supplemented with different carbon sources such as glucose, glycerol, pectin and PGA were 1.3, 2.4, 0.2 and 0.1 units/ml. But in the prescence of 1 mM $CaCl_2$ the enzyme levels increased to 4, 4.8, 5.2 and 5.44 U/ml respectively. However, there was no increase in enzyme production with the addition of 1 mM $CaCl_2$ in the medium containing glucose + PGA.

V

Ecology and epidemiology

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Molecular epidemiology of *Xanthomonas campestris* pv. *manihotis* causal agent of cassava bacterial blight

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Abstract

In order to detect and assess genetic and evolutionary relationships among strains of *Xc* pv. *manihotis* a comparison of strains of distinct geographical origin, representing 18 countries, was performed using a range of assays including restriction fragment length polymorphism (**RFLP**) analysis. The probes used were: 16+23S rRNA genes from *E.coli* and three restriction fragments from the chromosomal or plasmid DNA of *Xc* pv. *manihotis*.

Hybridization with the probe corresponding to the rRNA genes allowed the distinction of four RFLP groups. Subgroups were identified based on hybridization profiles with the three others probes.

Genetic variability of *Xc.* pv. *manihotis* was extensive in strains from the area of origin of the host plant and limited elsewhere. These results are in agreement with the hypothesis of the recent introduction of the pathogen to these latter areas and suggests that the African strains have not yet diversified genetically at the chromosomal level.

Our results indicate that RNA and DNA probes are useful tools for epidemiological studies and in following the genetic evolution of strains.

Keywords: *Xanthomonas campestris* pv. *manihotis*, cassava, RFLP, rRNA probe, DNA probe.

I. INTRODUCTION

Cassava (*Manihot esculenta*), family *Euphorbiaceae* is a root stock crop native from South and Central America. Portuguese traders introduced it to West Africa in the sixteenth century and to East Africa in the eighteenth century (SILVESTRE & ARRAUDEAU, 1983). It became one of the most important tropical food in countries of Tropical Africa.

Cassava bacterial blight (C.B.B) caused by *Xanthomonas campestris* pv. *manihotis* is one of the most important diseases of cassava. The disease was first reported in Brazil in 1912 (BONDAR, 1912) but has also been observed in Colombia and Venezuela (LOZANO & SEQUEIRA, 1974), as well as in most of African (MARAITE & MEYER, 1975) and Asian countries (BOOTH & LOZANO, 1978).

Cassava originated from South America and its related bacterial pathogens could have been propagated to others countries through the cuttings and seeds. To be able to detect and assess evolutionary relationship among pv. *manihotis* a comparaison of strains was developped using a wide range of assays.

II. MATERIAL AND METHODS.

X.c. pv. *manihotis* collection.

The bacterial strains used in this study, their geographical origin and their sampling collecting places are listed in Table 1.

Physiological characteristics.

Different phenotypic features were examined: the *in vitro* susceptibility to 20 antibiotics was determined, the utilization of carbon sources (19 tested), and the amylase activity according to described methods (GROUSSON et *al*, 1990).

Phytopathogenicity test.

Pathogenicity of all strains was tested on cassava plants, Congo's cultivar PMB, multiplied from cuttings. The stem inoculation was done according to previously described methods (MARAITE et *al*, 1981).

Table 1 : *Xanthomonas campestris* pv. *manihotis* strains used and information on their origin and isolation.

Strain no * and in other collection	Place	Year of isolation	Isolated y
CFBP1851,CIAT1111	Colombia	1974	Lozano.J
LMG 776, NCPPB2443,HMB72,CFBP2603		1970	Lozano.J
ORST1, CIAT1060, CFBP1849		1970	Lozano.J
ORST2, CIAT1061, CFBP1850	Venezuela	1971	Lozano
ATCC 23380, HMB68,NCPPB1159	Brazil	1941	Burkholder
HMB 70, NCPBB1160, LMG5273		1941	Drumond Hipolito
HMB 55a,NCPBB1834*, LMF784		1965	Robbs C.
ORST7, CFBP1854		1973	Neto J.R.
HMB23, LMG770		1973	Pereira A.
ORST3, CIAT 1120, CFBP1852		1974	Lozano.J
ORST5, CFBP1855		1974	Neto J.R.
ORST6, CFBP1856		1976	Neto J.R.
HMB79, LMG778		1978	Takatsu.A.
LMG777, HMB78		1978	Takatsu.A.
LMG779, HMB80		1978	Takatsu.A.
HMB25, NCPBB3060, LMG 771	Nigeria	1976	Maraite H.
ORST42		1978	Daniel J.F
ORST43		1978	«
CFBP1857, ORSTOM A202.1		1978	«
CFBP1858, ORSTOM A203.1		1978	«
CFBP1859, ORSTOM A205.1		1978	«
CFBP1860, ORSTOM A207		1978	«
ORST34	Benin	1982	Daniel J.F
ORST35		1982	«
ORST36		1982	«
ORST37		1982	«
ORST38		1982	«
CFBP1944	Ivory Coast	1979	Ridé M.
LMG5249, HMB203		1981	Maraite H.
ORST55		1984	Daniel J.F
ORST56		1984	«
ORST (198 strains)	Congo	1977-1991	Daniel J.F Boher B. Verdier V.
ORST (29 strains)	Togo	1987-1991	Boher B.
HMB6, LMG 767	Zaïre	1973	Maraite H.
HMB9, LMG 768, NCPBB3O58		»	»
LMG 769, NCPPB3O59, HMB10		»	»
LMG 766, HMB3		»	»
ORST44		1979	Daniel J.F
ORST45		»	»
ORST46		»	»
ORST47		»	»
ORST48		»	»
ORST49		»	»
ORST50		»	»
ORST51		»	»
ORST52		»	»
ORST53		»	»
ORST54		»	»
ORST186		1987	Boher B
ORST187		»	»
ORST39	RCA	1977	Daniel J.F
ORST40		»	»
ORST41		»	»
LMG 5287, NCPPB 3161	Cameroon	1976	Persley G.
HMB27, LMG629		1977	Maraite H.
LMG780, HMB81	Uganda	1979	Maraite H.
LMG782, HMB93		»	»
LMG783, HMB148	Kenya	1979	Maraite H.
LMG5288, NCPPB 3194	Niger	1978	Bradbury.J
LMG765	Malaysia	1980	Lan G.
LMG774, HMB60	Taïwan	1978	Maraite H.
HMB71, NCPBB1161, LMG775	Mauritius	1946	Orian G.
CFBP2624	Reunion	1986	Pruvost O.
CFBP2635		1987	Girard J.C

ATCC : Americain Type Culture, Rockville, Maryland, USA. **CFBP** : Collection Française de Bactéries Phytopathogènes, Angers, France.
NCPPB : National Collection of Plant Pathogenic Bacteria, Harpenden, U.K. **HMB** : H. Maraite's Bacterial Collection, LOUVAIN La Neuve, Belgium.
LMG : Laboratorium voor Microbiologie Gent culture Collection, Gent, Belgium. **ORST** : Collection du Laboratoire de Phytopathologie, ORSTOM, Brazzaville, Congo.
CIAT : Centro International de Agricultura Tropical, Cali, Colombia. * : Pathovar reference strain.

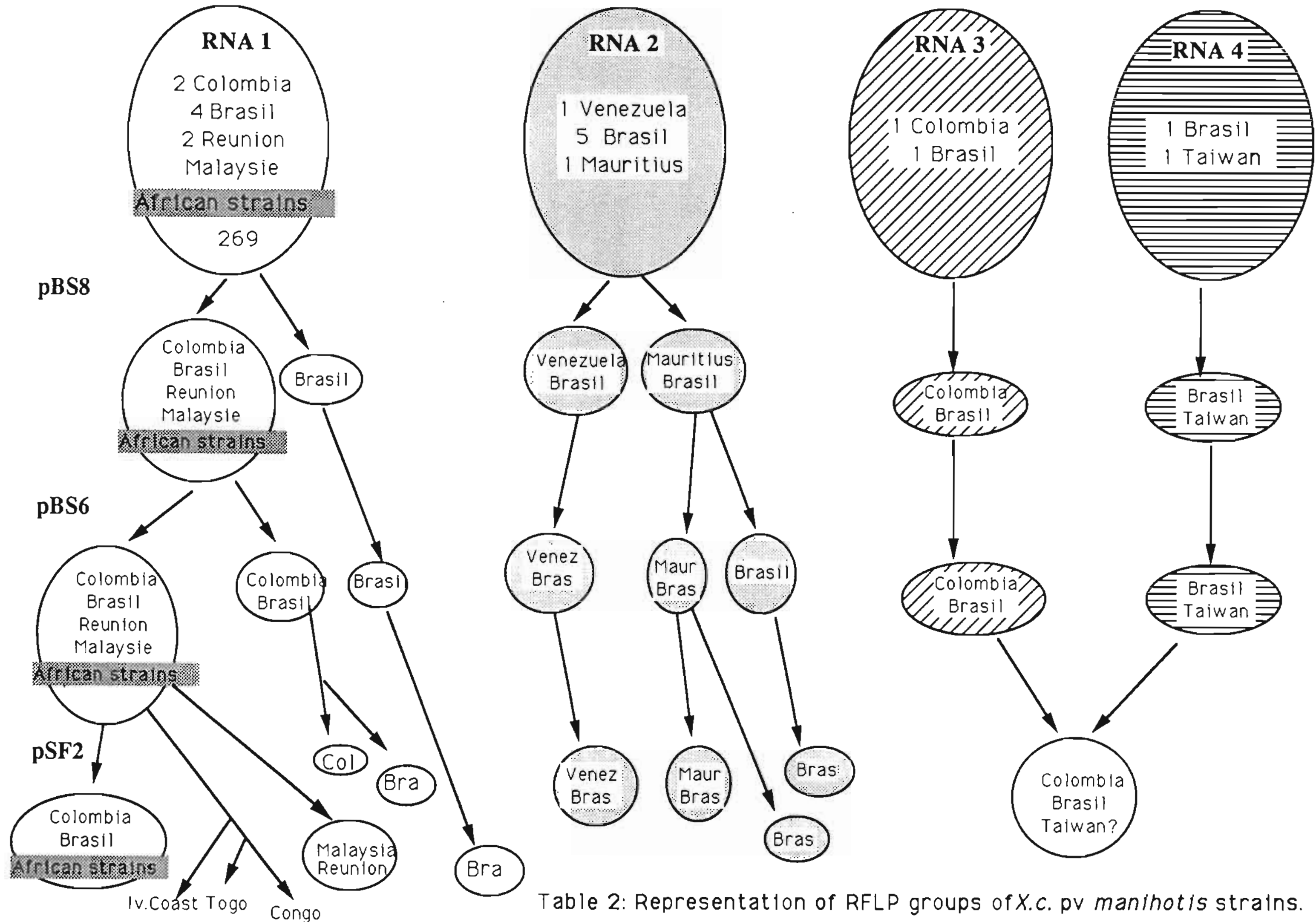

Table 2: Representation of RFLP groups of *X.c.* pv *manihotis* strains.

RFLP analysis.

Total genomic DNA isolation, endonuclease digestion, electrophoresis and Southern blot were done according to previously described methods (BERTHIER et *al*, 1992). Hybridization was made with different probes. Acetyl Amino Fluorene labeled ribosomal 16+23S RNA genes from *E.coli* (Eurogentec, Liege, Belgium) hybridized with the genomic DNA of bacteria. The rRNA - rDNA duplexes were detected using the anti-AAF monoclonal antibody (GRIMONT et *al*. 1989).

The DNA probes used in this study were: **BS6** (7kb-*Eco*RI) and **BS8** (8kb-*Eco*RI), two restricted fragments from the chromosomal DNA (*Xc.* pv *manihotis* strain CNBP1851-CIAT1111) and **pBsF2** derivated from the 13kb-*Hin*dIII fragment of plasmid DNA cloned in the bluescript vector plasmid. DNA probes were labeled *in vitro* by using a random priming kit with ^{32}P deoxycytidine triphosphate (Multiprime Amersham).

III. RESULTS

RFLP patterns.

Using the rRNA probe, the distinction of 4 RFLP groups among the 290 strains tested could be possible. Strains from South America were heterogenous and gave different patterns, on the contrary no polymorphism was noticed in African strains (Table 2).

Hybridization profiles with DNA probes could differentiate 6 groups with BS8 probe and 8 groups with BS6 probe, each group representing strains with identical RFLP pattern (Table 2). Polymorphism could be noticed in South American strains which are represented in groups mentioned above. In contrast, no polymorphism was observed in African strain with BS8 and BS6 probes suggesting that these regions are well conserved into the genome.

Variability among RFLP patterns of African strains was only noticed with the plasmid DNA probe pSF2.

Pathogenic characteristics.

Variability among pathogenic characteristics exists but was not related with the geographical origin of strains.

Phenotypic features.

Same results were obtained for two of the three phenotypic features tested (sensitivity to antibiotics and utilization of carbon sources). Starch hydrolysis was observed for all strains but two groups were differentiated. All African Reunion and Malaysian strains showed a low amylase activity similar to that found in 3 Brasilian and Colombian strains.

IV. DISCUSSION

Based on numerical analysis of protein gel electrophoregrams and, 267 phenotypic features, VAN DEN MOOTER et *al*, (1987) and VAUTERIN et *al*, (1991) indicate that the pv *manihotis* strains constitute a phenotypically and genetically homogeneous group. In this study, using the RFLP analysis, small changes in DNA organization were observed. Genetic variability of pv *manihotis* was more extensive in strains from the area of origin of the host plant and more limited in those coming from elsewhere. Among African strains homogeneity was observed with the probe corresponding to the rRNA genes and thus was confirmed with genomic probes used in this study. In our previous data based on plasmid DNA study we have indicated the hypothesis of one common geographic origin within strains of *Xc.* pv. *manihotis* (VERDIER, 1988). The results presented here agree with the hypothesis of the recent introduction of this pathogen from South America to the other countries, and suggest that African strains are not already diversified at chromosomal level. Using the DNA plasmid fragment as a probe, this study revealed that DNA polymorphisms exist in African strains. Plasmids are mobile elements which easily perform genetic exchange in bacterial strains (COPLIN, 1989; EBERHARD, 1990). Presence of essential pathogenicity genes on these plasmid fragment was previously demonstrated (VERDIER et *al*, 1989).

RNA and DNA probes used here were particularly useful in our epidemiological studies, providing information on the genetic population structure of these pathogens and its ability to identify clonally related individuals.

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Relation of *Erwinia carotovora* subsp. *carotovora* and *Erwinia carotovora* subsp. *atroseptica* with bacterial movement and tuber colonization in *Solanum* species

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ABSTRACT

The movement of Ecc and Eca was studied in stems of *S. brevidens, S. lycopersicoides, S. maglia, S. fernandezianum, S. tuberosum* (cv. Desiree), *S. rickii* a and *S. eutuberosum*. Bacterial counts were performed up to 30 days after inoculating plants, with 50 µl of a 4 x 10^8 CFU/ml cell suspension of chloramphenicol and rifampicin resistant isolates of Ecc and Eca.

The response of 10 potato cultivars to micro-inoculation with *Erwinia carotovora* subsp. *carotovora* (Ecc) and *E. c.* subsp. *atroseptica* (Eca) was studied by stem injecting 20 µl (O.D. = 1.0 at 600 nm) of a bacterial suspension of each subspecies to 60 days old plants. Thirty days after harvesting, all tubers were collected, surface sterilized and rot-induced and the relative weight loss (RWL) was determined after 7 days of incubation at 20, 28 and 36°C.

Results showed that at 20°C the RWL is low in all 10 cultivars studied and that this value increases at 28°C, being high at 36°C. Significant differences were determined at 28°C and 36°C, Pimpernel and R84521-4 being the two cultivars that showed the lowest RWL values. Populations counts of Ecc and Eca in stems of *S. lycopersicoides, S. brevidens, S. maglia, S. fernandezianum* and *S. tuberosum* showed increasing values up to the second day and then counts sharply decreased, except for *S. tuberosum* whose populations showed slight increased values up to 32 days after inoculation. In *S. brevidens* and *S. maglia* the CFU/ml of Ecc and Eca started to decrease after day 2 of sampling and then populations were lower than in other *Solanum* species. *S. rickii* and *S. tuberosum* counts of Ecc and Eca showed a tendency to increase slightly 32 after inoculation.

KEYWORDS

Soft rot, blackleg, *Erwinia*, *Solanum*, resistance.

INTRODUCTION

Potato is among the most important dicotyledonous plants used for human consumption, being overcame only by wheat, rice, corn and barley (VAN DER ZAAG, 1976). Potato is also important for its food value, mainly due to the protein biological value, high vitamin C content and presence of minerals (KALDY, 1972). For Chile potato is the second most important crop because of its yield value and job generation, playing an important role in the chilean diet because the energy content and protein value (MONARES *et al.*, 1988).

The southern part of Chile is responsible for 40% of the total potato production of the country. In this area, disease losses are most important and are present in both plants and tubers, in the field, during storage and transit. Among agents responsible for decreasing potato productivity is the soft rot *Erwinia* (Ecc and Eca) (CIAMPI *et al.*, 1991). Also, Chile is the sub-center of origin of the modern cultivated potato (*Solanum tuberosum* subsp. *tuberosum*) and in many areas of the country plants of the genus *Solanum* are found growing wildly. By the other hand, cultivated potato and its wild relatives present a wide genetic basis, fact that may play an important role in breeding for resistance to control diseases such as soft rot.

The purpose of this research was .

a) to study the bacterial movement of Ecc and Eca in stems of several non cultivated *Solanum* species
b) to study the tuber response of field inoculated *S. tubesorum* selected clones with Ecc and Eca.

MATERIAL AND METHODS

Study of the bacterial movement of Ecc and Eca in stems of several non cultivated *Solanum species*

The following *Solanum* species were used : *S. brevidens, S. lycopersicoides, S. maglia, S. fernandezianum, S. tuberosum* cv. Desiree, *S. rickii* and *S. eutuberosum.* All the plants grown from stem cuttings maintained on pots and kept under greenhouse conditions were provided by the Potato Germplasm Bank of Austral University of Chile. Two isolates of *Erwinia* were used, E7 (Ecc) and E8 (Eca), they were obtained from the Culture Collection of the Bacteriology Section of the Institute of Plant Protection and Production, Austral University of Chile. For an easy recovery of Ecc and Eca inoculated to *Solanum* plants, antibiotic resistant strains were obtained according to MILLER (1972).

Twenty plants each of *S. lycopersicoides, S. brevidens, S. maglia, S. fernandezianum* and *S. tubesorum* cv. Desiree, were stem inoculated separately with Ecc and Eca isolates in the insertion site of the third leaflet with 50 µl of a suspension of 4 x 10^8 CFU/ml (O.D. = 1.0 at 600 nm). The inoculation was done by inserting into the stem a micro capillary tube with the bacterial suspension, leaving the tube inserted during all the growing season. The same procedure was used to stem inoculate 10 plants of each *S. rickii* and *S. eutuberosum*. All the plants were kept under greenhouse conditions during the experiment and stem samples were taken 5 cm below the inoculation site at 0, 1, 2, 3, 5, 10, 15, 20, 25 and 30 days for all the *Solanum* species except for *S. eutuberosum* and *S. rickii* that were sampled at 0, 4, 8, 16 and 32 days after inoculation.

Tuber response of field inoculated *S. tuberosum* selected clones with Ecc and Eca

The following cultivars and clones were field inoculated using the same procedure described in the previous section : Desiree, Yagana, Ultimus, Serrana, Pimpernel, Atzimba and the clones C788, R82139-1, R82248-b-1 and R84521-4. Plants were provided by the Instituto de Investigaciones Agropecuarias (INIA), Remehue Experimental Station (Osorno, Chile). Potatoes were planted during November 1990, arranged in a randomized block design with six replicates. Ten tubers were planted in each block. Plants were stem inoculated with 20 µl of bacterial suspension of antibiotic resistant Ecc and Eca two months after planting.

At harvesting time all the daughter tubers were collected and gathered in three groups and 30 days after the tubers were evaluated as follows : each tuber was surface sterilized during 15 min with 10% sodium hypochlorite (w/v), air dried, weighted and wrapped with moist sterile paper towel and alusa plast. Potato rot was induced at 20, 28 and 36°C for an incubation period of 7 days. After this period, rotted tissue was removed from each tuber, air dried and finally weighted. For statistical analysis the difference between starting potato weight and final weight was used and the percentage was calculated (Relative Weight Loss = RWL). The Duncan Multiple Range Test was used to compare means for each temperature and for all the cultivars tested.

RESULTS

In *S. lycopersicoides, S; brevidens, S. maglia, S. fernandezianum* and *S. tuberosum* cv. Desiree the CFU/ml were high at day 2. After this moment counts started to decrease sharply and at day 30 counts were undetectable except for *S. tuberosum* cv. Desiree, in which counts of Ecc were detectable (400 CFU/ml) and Eca was still high (21.350 CFU/ml).

DISCUSSION

The response of several *Solanum* species to stem inoculation with Ecc and Eca follows the tendency of independent action and co-operative action as was described by ERCOLANI (1973). The homologous host for both Ecc and Eca is *S. tuberosum* and both subspecies are still present in stems of potato plants 30 days after inoculation. The contrary was established in the other inoculated *Solanum* species in which bacterial population of both Ecc and Eca clearly have a tendency to decrease and become undetectable. This confirm work conducted by others which states that *S. tuberosum* normally shows a susceptible reaction toward Ecc and Eca. This fact also indicates the difficulties in finding plant or tuber resistance to bacterial soft rot in *S. tuberosum*

The response to bacterial population in *S. eutuberosum* and *S. rickii* was similar to the response observed in *S. tuberosum* but the size of the populations was lower. This could be related to a genetic closeness of this two *Solanum* species to *S. tuberosum*. The contrary can be speculated since much lower or no populations were detected in *S. lycopersicoides* and *S. fernandezianum*, plant species that may have an important role in transferring genetic resistance to *S. tuberosum*.

Previous studies conducted by CIAMPI & ANDRADE (1984) showed the importance of some *S. tuberosum* clones that showed tuber resistance to Ecc and Eca. However, tuber inoculation alone may be not a reliable system to detect resistance to *Erwinia*. In fact resistance to *Erwinia* is being studied in hexaploids hybrids such the somatic cross between *S. brevidens* (2X) and *S. tuberosum* (4X) (AUSTIN *et al.*, 1987). By the other hand, high level of resistance to blackleg has been found in *S. chacoense* and *S. stoloniferum* (DOBIAS, 1977). Also LOJKOWSKA & KELMAN (1989) determined that many genetic lines of *S. berthaultii, S. bulbocastanum, S. chacoense, S. stoloniferum, S. tarijense* and *S. tuberosum* ssp. *andigenum* were very resistant to stem rot. Therefore, it could be possible to use come of the wild non cultivated *Solanum* species tested in this study to transfer resistance to *S. tuberosum*. Especially interesting are *S. fernandezianum* which is found in the Robinson Crusoe Islands and *S. lycopersicoides* that grows in the north part of the country.

Rot induction of potato tubers obtained from stem inoculated plants with Eca and Ecc showed high susceptibility to tuber rot, especially at 36°C. Evidence indicated that bacteria can move from the inoculation site to daughter tubers and establish latent infections. A similar model was described for *Pseudomonas solanacearum* (CIAMPI & SEQUEIRA, 1980 ; CIAMPI *et al.*, 1980). The different temperatures used to induce rot indicated that Eca and Ecc must be tested at the same temperatures than those normally acting under field or storage conditions. The temperature of 20°C is insufficient to induce rot. Being, 28 and 36°C the proper testing temperatures for Eca and Ecc respectively.

For *S. brevidens* and *S. maglia* the CFU were high the first two days, but, from then, populations started to decline. A 30 days counts of Ecc and Eca were undetectable. The populations of Eca were high at the beginning of the experiment in *S. maglia*, *S. brevidens*, *S. lycopersicoides* and *S. fernandezianum* started to decrease after a maximum value at day 2 and were undetectable at day 30.

In *S. rickii* and *S. eutuberosum*, the presence of low amounts of both Eca and Ecc was possible to observe one day after inoculation but the populations size was very small although still detectable at day 32. In *S. eutuberosum* counts of Ecc after 32 days were 8.950 CFU/ml and for Eca were 7.675. By the other hand in *S. rickii* populations of Ecc were 550 and Eca 2.200 CFU/ml at day 32.

The RWL for each potato cultivar tested at three different temperature is presented in Table 1. It is possible to establish three kinds or groups of clones that have significant differences. One group, the larger, showed a high susceptibility to Ecc and Eca, having a RLW larger that 26%, a second group, with a RLW between 26 and 4% and a small group with lower than 4% values. Taking under consideration the temperatures, at 20°C the amount of rot induced in tubers by Ecc and Eca was low, this value increased at 28°C being higher at 36°C. At 28°C only the selected clone R84521-4 showed a high resistance. Clone R82248-b-1 and variety Atzimba showed low resistance and the rest had an intermediate value. At 20 and 28°C Eca was mostly detected as rotting agent whereas at 36°C Ecc was detected in 100% of the tubers and Eca in 14%. At higher temperatures, different response among the several clones tested was more clearly detected. Cultivar Pimpernel and clone R84521-4 showed high resistance. Cultivars Yagana and Serrana showed intermediate response and the rest presented high values of RWL.

TABLE 1. **Relative weight loss (RWL) of tissue determined in rot-induced potato tubers harvested from Ecc and Eca field inoculated plants.**

CULTIVAR	TEMPERATURE °C		
	20	28	36
R82139-1	0,02±0,04	2,5±0,6 b	60,9±6,0 d e
R82248b1	0,00	47,9±8,0 e	55,1±31 e
ATZIMBA	0,00	47,9±3,5 e	52,3±14 e
DESIREE	0,16±0,1	6,1±1,6 c	46,2±11 e
C788	0,06±0,02	7,2±2,0 c	45,9±4,1 c e
ULTIMUS	0,32±0,1	14,3±4,7 d	35,8±7,0 c e
SERRANA	0,95±0,5	12,0±2,6 d	22,3±3,4 b
YAGANA	0,09±0,05	10,8±3,2 c d	19,6±1,6 b
R84521-4	0,00	0,96±0,2 a	1,1±0,05 a
PIMPERNEL	0,00	11,8±5,9 c d	0,4±0,1 a

Within a column, any two means followed by a common letter are not significantly different at p= 0.01.

Among the clones and cultivars tested, clone R84521-4 and cv. Pimpernel showed the lowest amount of tissue losses. It is interesting to note that clone R84521-4 was created in crossing parental lines resistant to *P. solanacearum* obtained from *S. phureja*, native genes from cv. Yagana and clone H412-1 derived also from *S. phureja**. *At 28°C, beside, clone R84521-4 also clone R82139-1, cv. Desiree and clone C788 had the lowest amount of tissue losses. At 36°C cv. Pimpernel*, clone R84521-4 and cv. Yagana and Serrana showed the lowest amount of tissue losses. These differences could be related with the optimum temperatures that Ecc and Eca need to induce rot.

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Spread of *Pseudomonas syringae* pathovar *phaseolicola* over the bean foliage and effect on seed contamination

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ABSTRACT

The spread of *P. s.* pv. *phaseolicola* strain SD68 was monitored all along the growing season on the foliage of bean cultivar Michelet in a plot (6 m x 12 m). One fourth of the plot (6 m x 3 m) was inoculated by spraying a bacterial suspension (1 x 10^7 cfu/ml) on bean at primary leaf stage (July 16th, 1991).

The strain dispersed rapidly all over the plot. Four to 20 % of leaves were contamined in the distal half part of the plot within one month. The maximum level of contamination, 60 % of leaves contaminated by more than 10^3 cells per leaf, was reached on august 27th and remained constant throughout the end of the culture.

The contamination of seed was monitored after harvest. The sub plot inoculated by spraying gave seeds highly contaminated (50 to 100 % of seeds, 1.53 x 10^6 to 3.6 x 10^6 bacteria/seed). Despite the foliage had been highly contaminated since the blooming period in the distal half part of the plot, the seeds were much less contaminated (0 to 20 %, 0 to 45 bacteria/seed).

These results suggest a new strategy to control the halo blight disease : it could be possible to predict seed contamination by checking the occurrence of *P. s. phaseolicola* on leaf.

KEY-WORDS : *Pseudomonas syringae* pathovar *phaseolicola*, bean, foliage contamination, seed contamination

INTRODUCTION

Pseudomonas syringae pathovar *phaseolicola* cause halo blight of beans. It is a typical seed obrne pathogenic bacterium and primary infections are due to infected seeds. A very low proportion of infected seeds (TRIGALET & BIDAUD, 1978) may cause severe oubreak of the disease in the field. Plant contamination has been usually assessed by symptoms monitoring in the fields, out contaminated seeds are sometimes recovered from fields having no unhelp eye detectable symptoms.

The purpose of the research reported here is to examine the multiplication of *P. s.* pv. *phaseolicola* on bean foliage all along the growing season and to assess the incidence of bacterial colonization of leaves on seed contamination.

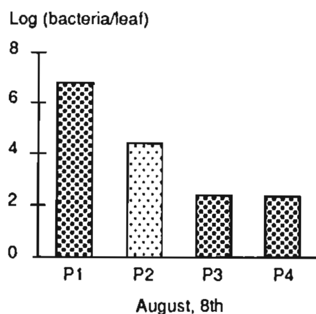
MATERIALS AND METHODS

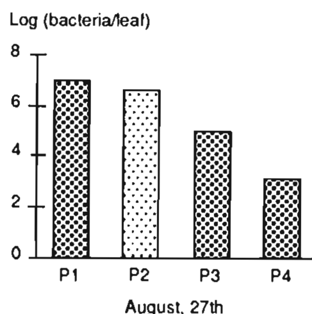
The experiment was conducted in Angers area, France in 1991. Beans cv. Michelet were grown in an experimental plot (12 m x 6 m). Thiks plot was divided into four subplots (3 m x 6 m). Plant of one of the distal subplot were inoculated by the pathogenic strain SD68. A bacterial suspension in sterile distilled water (1 x 10^7 cfu/ml) was sprayed on primary leaf on July 16th. The plot was irrigated by sprinkler aspersion twice a week for 1 hour. Bacterial populations were assessed on symptom-free trifoliate leaves by grounding then in sterile distilled water and pleting dilutions on semi selective medium MSP (MOHAN & SHAAD, 1987-. Eleven samples were collected periodically in each subplot (1 x 50 leaves, 5 x 5 leaves, 5 x 1 leaf) average numbers of bacteria per leaf were calculated for each subplot and the percentage of leaves bearing bacteria were assissed using the most probable number (MPN) method (SWAROOP, 1951). After havest (Sept., 20th) seeds were tested for bactrial contamination. 44 samples of seed were analysed for each subplot (4 x 1000seeds, 20 x 100 seeds, 20 x 10 seeds) the average numbers of bacteria per seed and the percentage of infected seed were assessed.

RESULTS

• *Contamination of symptom free leaves*

Leaf contamination occurred very rapidly in the inoculated subplot : 6,6 x 10^6 bacteria per leaflet were recoovered 23 days after inoculation and





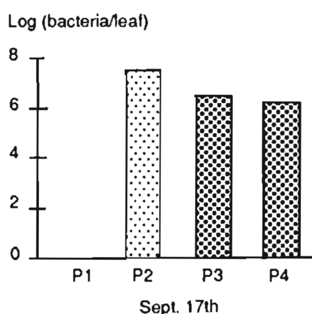


Figure 1 : average numbers of *P. s.* pv *phaseolicola* strain SD68 per leaflet in the experimental plot 23 (August 8th), 42 (August 27th) and 63 days (Sept. 17th) after inoculation respectivally. The experimental plot was divided into four sub plot (P1, P2, P3, P4). The primary leaves of beans located in sub-plot P1 were inoculated with a bacterial suspension 1.10^7 cfu/ml of *P. s.* pv *phaseolicola*.

sampling date	sub plot	colinized leaves %
August 8th (23 days)*	2	28 ± 12
	3	16 ± 4
	4	12 ± 6
August 27th (42 days)	2	60 ± 40
	3	40 ± 20
	4	46 ± 34
September 17th (63 days)	2	60 ± 40
	3	50 ± 30
	4	40 ± 20

table 1 : percentage of bean leaves colonized with *P. s.* pv *phaseolicola* in experimental plot assessed by the MPN method at each sampling time.

* number of days after inoculation

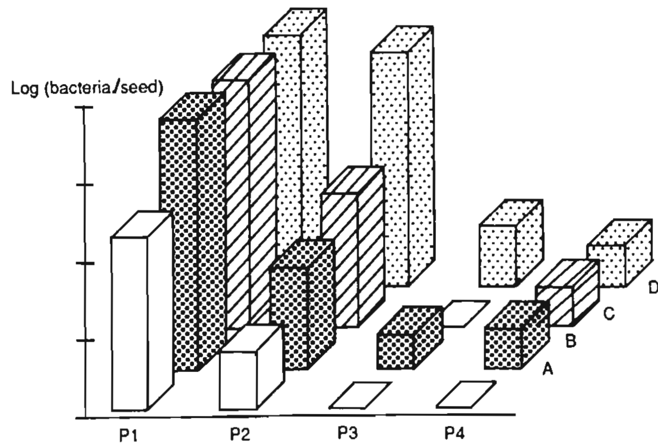


Figure 2 : average number of *P.s.* pv *phaseolicola* strain SD68 per seed after harvest. 4 X 5 samples of 100 seeds were analysed from each sub plot P1, P2, P3, P4.

sub plot	infected seeds %
1	47.5 ± 42
2	10.63 ± 9
3	2.65 ± 2
4	0.5 ± 0.5

Table 2 : percentage of seed contaminated with *P. s.* pv *phaseolicola* after harvest.

still increased by the end of the growing season. Nevertheless, to many symptoms occurred in this subplot at the harvest time to make a significant estimation of population on symptom-free leaves (fig. 1).

P. s. pv. *phaseolicola* spread gradually over the foliage of the plot (fig. 1). Twenty three days after inocution (Aug. 8th) leaves of the closest subplot from the inoculated beared an average of 2.45 x 10^4 cfu per leaflet when leaflets of the two distal plots beared only 2.3 x 10^2 cfu per leaflet. Whereas, progressively, population of *P. s.* pv. *phaseolicola* increased in all subplot and finally more than 1.5 x 10^6 cfu ml per leaflet were recovered in all the experimental plot.

The assesment of the percentage of leaf bearing *P. s.* pv. *phaseolicola* showed the same progression of the bacteria in non-inoculated subplots than the assesment of the number of bacteria per leaf. More than 50 % leaves were contaminated by *P. s.* pv. *phaseolicola* by the end of the culture in all subplot (table 1).

· ***Contamination of seeds***

Contaminated seed were recovered in all four subplots. However, the level of contamination of seeds decreased steeply along the experimental plot as it is shown by the assesment of the number of bacteria per seed (fig. 2) and by the assesment of the percentage of contaminated seed in each plot as well (table 2).

DISCUSSION-CONCLUSION

Irrigation of plants by overhead sprinklers twice a week created conditions especially favorable for the dissemination of *P. s.* pv. *phaseolicola* and development of halo blight. Previous studies showed the importance of the climatic condition on symptoms appearance (WALKER & PATEL, 1964 ; KATHERMAN *et al*, 1980). We found that a high population of *P. s.* pv. *phaseolicola* occurred even on symptoms free leaves.

Although, foliage contamination were homogeneous by the end of the growing period of bean plants, the contamination of seed were decreasing. In the farest subplot the seed contamination relatively low also the leaf contamination was an high as in very contaminated subplot. These results suggest that seed contamination could occur early in the growing season.

Despite the climatic conditions especially favorable for bacterial multiplication on leaves, it should be emphasized that the number of *P. s.* pv. *phaseolicola* on leaves at harvest time made the detection of the occurrence of the pathogenic bacteria easier than on seed.

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Ice nucleation activity of *Pseudomonas viridiflava* and its importance in frost injury to kiwifruit

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Abstract
A hundred and forty-one isolates of *Pseudomonas viridiflava* were obtained from diseased kiwifruits (*Actinidia deliciosa*) and from the surfaces of leaves, shoots, floral buds, flowers and fruits of symptomless plants growing in the central area of Italy. Thirtyfive percent of the bacterial isolates induced ice formation at temperatures higher than -5°C, and a third of these INA bacteria were active in ice formation at temperatures above -3° C. Suspensions of INA strains of *P. viridiflava* sprayed onto leaves of kiwifruits increased the sensitivity of plant tissues to frost injury. The severity of damage was related to: *(i)* bacterial concentration, *(ii)* relative humidity, *(iii)* time of incubation between spraying and freezing.

Keywords: INA, *Pseudomonas viridiflava*, frost injury, kiwifruit.

Introduction

Pseudomonas viridiflava (Burkholder) Dowson is the causal agent of bacterial blight of kiwifruit [*Actinidia deliciosa* (A. Chev.) Liang *et* Ferguson]. In literature it is reported that the pathogen is able to survive on kiwifruit phylloplane (YOUNG *et al.*, 1988) and that some of its isolates are active catalysts for ice formation.

During surveys carried out from 1988 to 1990 (VARVARO *et al.*, 1990), 141 isolates of *P. viridiflava* were obtained from diseased kiwifruits and from the surfaces of leaves, shoots, floral buds, flowers and fruits of symptomless plants growing in the central area of Italy.

The objective of this study was: *(i)* to evaluate the ice nucleation activity (INA) of the *P. viridiflava* isolates; and, *(ii)* to define their role in frost damage to plants.

Materials and methods

Droplets of bacterial suspensions of each isolate containing $3x10^8$ cfu/ml, were placed in a glass tray floating on a refrigerated bath. Temperature was decreased from 0°C to -10°C at intervals of 0.5°C. Freezing of droplets was observed visually. The ice nucleation spectrum of those bacterial isolates with INA at -3°C was determined by testing tenfold dilutions of each suspension at -3°C.

The bacterial isolate that showed better activity in ice nucleation was utilized in the frost injury essay. Two bacterial concentrations were sprayed on young potted kiwifruit plants: 10^4 and 10^7 cfu/ml. Plants were incubated in a mist chamber (>80% RH) or in ambient air (<50% RH) before freezing.

Immediately, 2 h or 48 h after spraying plants were cooled from +5°C to -3°C at 0.1°C/min. Plants remained at -3°C for 30 min.

Results

Forty-nine isolates (about 35%) of *P. viridiflava* were able to nucleate at temperature ≥-5°C and 17 of these nucleated at -3°C. When diluted suspensions of the 17 isolates were tested, it was seen that at higher dilutions less bacterial isolates were active in ice nucleation.

Kiwifruit plants sprayed with *P. viridiflava* and exposed to freezing were frost injured more seriously when the bacterial suspension contained 10^7 cfu/ml and when the cooling occurred immediately after spraying or 48 h after spraying and incubation of plants in mist chamber.

Discussion

The results of this study showed that: *(i)* an interesting percentage of isolates of *P. viridiflava*, obtained from different orchards of central Italy, are active in ice nucleation at temperatures higher than -5°C, according to YOUNG (1987); *(ii)* when *P. viridiflava* was sprayed on kiwifruit plants it was able to incite frost injury, more severe when the RH values were high.

In Latium (central Italy), where kiwifruit is one of the most important crops, it was observed (BALESTRA, 1991) that *P. viridiflava* is present on kiwifruit phylloplane with values ranging from 1.2×10^1 to 1.0×10^5 cfu/cm^2 of leaf. Moreover, it is not infrequent that temperature drops to -3/-5°C.

These considerations led to the conclusion that *P. viridiflava* may play an important role in frost injury to kiwifruit plants in the field.

Preventive treatments with copper compounds are necessary to reduce the epiphytic populations of ice nucleation active bacteria and therefore avoid frost injury.

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Detection of pathogenic *Agrobacterium* in soils by PCR

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Key words: *Agrobacterium*, Ti plasmid, detection, soil, PCR

ABSTRACT

A rapid method based on the polymerase chain reaction was developed to detect and enumerate pathogenic agrobacteria present in soils. Specific primers were characterized in the conserved *vir* genes of the Ti plasmid which permitted to differenciate pathogenic from non pathogenic cells of *Agrobacterium*. Specific detection and enumeration of inoculated *A. tumefaciens* strain was obtained in various soils, indicating the validity of the method for routine detections of pathogenic agrobacteria. The method was used to determine the density of pathogenic *A. tumefaciens* in a naturally contaminated soil.

INTRODUCTION

Agrobacterium tumefaciens is a soil bacteria which induces a major plant disease, crown gall, particularly prevalent in fruit tree nurseries. A good prophylaxis is merely obtained by the plantation of healthy plants. However, in order to avoid the plantation of healthy plants in contaminated soils, it is necessary to check the lack of pathogenic agrobacteria in soils. As no serum specific for pathogenic forms is presently available (NESME *et al.*, 1990), the usual method of detection of pathogenic agrobacteria consists in isolating *A. tumefaciens* and testing their pathogenicity on various plant species. This method is space and time consuming and was rather unsuitable for routine diagnoses of numerous samples, since the ratio of pathogenic to non-pathogenic forms of *A. tumefaciens* often reach only 1/500 in contaminated soils (MOORE & COOKSEY, 1981), and because several agrobacterial biotypes requiring different selective media generally co-exist in soils.

A. tumefaciens is pathogen only if it harbors a large plasmid, called Ti plasmid or pTi (ZAENEN *et al*, 1974). In that sense, the pTi is the true pathogen, and the recognition of contaminated soils results from the detection of Ti plasmids themselves. Molecular methods allow the recognition of DNA sequences specific of pTi, and, among those methods, the Polymerase Chain Reaction or PCR (MULLIS & FALOONA, 1987), permitted a fast discrimination of pathogenic and non-pathogenic *A. tumefaciens* isolates (NESME *et al.,* 1990 ; DONG *et al.,* 1992). PCR is also interesting to detect microorganisms in the environment since it requires only little DNA quantity of target DNA. PCR is a choice method for the detection in complex environment like soils (JOSEPHSON *et al.* 1991 ; PICARD *et al.* conditionally accepted by Appl. Environ. Microbiol.) but, for routine purpose, this need to be verified on several soils. The aim of this study was to check whether PCR allowed the specific detection of pathogenic *A. tumefaciens* cells inoculated in various soils. The method was then used to detect pathogenic *A. tumefaciens* in a naturally contaminated soil.

MATERIALS AND METHODS

Bacterial strain. The *Agrobacterium tumefaciens* strain C58, supplied by the CFBP (INRA Angers, France), was used as a seed organism.

Soil inoculation. Soil samples of 100 mg were inoculated with 10^6, 10^5, 10^4, 10^3 and 10^2 *A. tumefaciens* cells and washed in sterile distilled water. Inoculum densities were optically adjusted at 600 nm and verified by standard plate counts.

Bacterial enumeration by MPN-PCR. DNA extracted from soil solutions were serially diluted by using a factor of three and amplified by PCR in triplicate. The number of amplifiable target DNA sequences was determined according to the most probable number (MPN) technique.

Soil characterization. Five soils have been tested. Two silt loam soils, Lyon and La Toussuire, a sandy loam soil, Peyrat, a clay soil, Florence (Italy), and soils of the Rhône river valley, Mondragon 1 and 2.

Oligonucleotide primers. Two *A. tumefaciens* specific primers (44-*vir* primer: 5'-TGCCGCATGGCGCGTTGTAG-3' and 14-*vir'* primer 5'-GAACGTGTTTCAACGG TTCA-3') were characterized in the *vir* region located on the Ti plasmid and could amplify a 246 bp DNA fragment (NESME *et al.*, 1990). Amplification occurred for nopaline and octopine type pTi plasmids but not for pTi-free agrobacteria (NESME *et al.*, 1990).

Extraction, purification and PCR amplification of soil-DNA The extraction procedure consisted in a succession of three lytic treatments performed as previously described (Picard *et al.*, conditionally accepted by Appl. Environ. Microbiol.) : ultrasonication (4/10 maximum power of a 600 W-ultrasonicator, Bioblock, Illkirsh, France, for 5 min at 50% of active cycles), micro-wave heating (5 x 1 min, 900 W), and three successive thermal shocks (liquid nitrogen: -196°C/ boiling water: +100°C). DNA solution was then purified through a series of 3 successive Elutip d columns (Schleicher and Schuell, Dassel, FR-Germany) conducted as specified by the manufacturer. The PCR amplification is a biphasic protocol in which primer concentration was varied over the range 1 nM for the 10 first cycles to 0.1 µM for the following cycles.The 10 first PCR cycles were run according to the following protocol: Initial denaturation: 95°C, 3 min; cyclic denaturation: 95°C, 1 min; annealing: 55°C, 1 min, extension: 72°C, 1 min. At the end of this first series of cycles the primer concentration was adjusted to 0.1 µM and 60 new PCR cycles were run in which DNA was denatured at 90°C, for 1 min, annealed at 55°C for 1 min and extended at 72°C for 1 min. Positive amplification by this method requires only one target DNA sequence (PICARD *et al.*, conditionally accepted by Appl. Environ. Microbiol.).

RESULTS AND DISCUSSION

Efficiency of the method in detecting inoculated bacteria. PCR assays were performed using different dilutions of the DNA solutions as template DNA in order to determine a routine dilution factor suitable for every soil. When DNA solutions were diluted 1/1000, positive PCR amplifications were obtained for each soil, as indicated by the occurence of a 246 bp-long fragment in agarose gels (not shown). Inconsistent results were generally obtained with less diluted DNA extracts probably as the consequence of the presence of soils impurities (PICARD *et al.*, conditionally accepted by Appl. Environ. Microbiol.). Among soil impurities, humic compounds are known to strongly inhibit *Taq* polymerase (STEFFAN & ATLAS, 1988.). As a consequence, when using a low density of bacterial inoculum (i.e. 10^2 cells per 100-mg soil sample) positive amplifications were obtained.only with poorly inhibiting soils, such as the soil of Lyon, while, positive detections were consistently obtained for stronger inocula ranging from 10^3 to 10^6 cells per 100-mg- soil sample.

Soils were used to test pTi enumeration by PCR, since no amplification was obtained with non-inoculated samples, indicating the lack of detectable

pathogenic agrobacteria in these soils. 100-mg-soil samples were inoculated with 5 10^6 *A. tumefaciens* cells, prior to DNA extraction. The amount of target sequences in DNA extracts were then determined by using an adaptation of the MPN technique (Table 1). MPN-PCR determination were rather equivalent for 4 / 5 soils, suggesting that the efficiency of DNA extractions were equivalent in these four soils. The number of pathogenic agrobacteria determined by MPN-PCR was underestimated by a factor of about 10, probably because sonication broke numerous target DNA sequences (PICARD *et al.*, conditionally accepted by Appl. Environ. Microbiol.). Therefore, we must ten-fold multiply MPN-PCR data in order to obtain suitable estimations of the initial bacterial density.

Soils	MPN-PCR
La Toussuire	0.2×10^7
Mondragon	0.3×10^7
Lyon	0.8×10^7
Florence	0.3×10^7
Peyrat	0.4×10^6

Table 1: Enumeration by MPN-PCR of the number of PCR-amplifiable target vir sequences in various soils inoculated with 5 x 10^7 cells of *A. tumefaciens* C58 per gram of soil.

Efficiency of the method in detecting and enumerating naturally-occuring pathogenic agrobacteria.

DNA was extracted from soil samples harvested in a nursery known to suffer reccurent crown gall outbreaks. PCR performed with DNA extracts indicated the natural occurence of pTi, and thus probably of pathogenic agrobacteria in this soil. The level of amplifiable pTi sequences determined by MPN-PCR was 1.2 x 10^4 per 100-mg soil sample. In order to estimate the bacterial population, we used another soil (Mondragon 2) naturally not contaminated by *A. tumefaciens*, but harvested close to the diseased nursery (Mondragon 1). There was a 17-time underestimation of the *A. tumefaciens* population inoculated in the soil Mondragon 2. Thus, using this corrective factor, we estimate the density of pathogenic *A. tumefaciens* in the naturally contaminated soil of Mondragon 1 to about 2 x 10^6 bacteria per gram. Very few data are available on the level of pathogenic

agrobacteria in soils, but our results are in agreement with overall agrobacterial density provided by BOUZAR *et al.* (1987).

In conclusion, PCR appears as a rapid and reliable method to detect and enumerate pathogenic agrobacteria, which can be used with various soils. Precise enumeration requires, however, the determination of a factor that corrects for the loss of amplifiable DNA during steps of DNA extraction.

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The application of conductimetric assays in phytobacteriology

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Introduction

The conversion of uncharged or weakly charged substrates due to bacterial metabolism in a growth medium, generally results in a change of conductance in the medium. This conductance change can be measured directly in the medium (direct conductimetry) or e.g. in a KOH-solution which traps CO_2 evolved from the medium as result of bacterial metabolism (indirect conductimetry). Conductance measurements are used for rapid measurements of total microbial contamination of food products (MARTINS & SELBY,1980), for specific detection of human pathogens e.g. salmonellas (GIBSON, 1987; BOLTON, 1990) and for determining antibiotic sensitivity (HOGG *et al.*, 1987). For studies on plant pathogenic bacteria the application of conductance measurements is new and no information is available yet on suitable media, test formats and potential applications. In this study the effects of various growth conditions on conductance responses caused by some plant pathogenic bacteria was investigated. This paper shows some of the results.

Materials and Methods

Special Peptone Yeast Extract (SPYE) and Malthus Colombia Broth (MCB) were used for direct conductimetry. Indirect conductimetry was done with an 0.1% agar slant of a minimal medium containing 1% homoserine. Conductimetric assays were done with an automated system (Malthus 2000) using 8 ml or 11 ml cells for resp. direct and indirect conductimetry. The detection time (Td) is defined in the software by the manufacturer as the time at which three

consecutive conductance changes of 0.8 µS or larger appear; the Td is recorded, automatically. In practice, the Td should represent the time at which the conductance curves accelerate (first significant conductance change). In each case one ml of bacterial suspension was added to 2 ml of growth medium. Conductance measurements were recorded at 6 min intervals. The following test strains were used: *Erwinia chrysanthemi* 502 (Ech 502), *E. carotovora* subsp. *atroseptica* 161 (Eca 161), *Pseudomonas syringae* pv. *pisi* 518 (Pspi 518), *P. s.* pv. *syringae* 1147 (Pss 1147) and *Xanthomonas maltophilia* 1974 (Xm 1974).
For direct detection of Ech 502 and Eca 161 in peel extracts of potato, cv. Bintje, the bacteria were added in different concentrations to the peel extracts. One ml of spiked peel extract was added to 2 ml of Malthus Enterobacteriaceae Medium (MEM). All experiments were done at 27 °C, unless otherwise stated.

Results

Differences between bacteria, cell concentrations and temperature

Fig. 1 shows that the highest cell concentrations (10^7 cfu/ml) result in the lowest Td, and the lowest cell concentration (10^6 cfu/ml) in the highest Td. For all bacteria tested the Td was also inversely proportionate to the log cell concentrations. Conductance curves show the same slope and maxima for the same bacterial strain tested. The curves indicate by their detection times and slopes that Eca 161 is metabolically more active at 27 °C in SPYE than Pspi 518. The curves for Pspi 518 also indicate that the Td (+) as used by the software is not optimal, because a significant acceleration of the curve starts much earlier than the time indicated.

Fig. 2 shows that as a result of a lower temperature, 17 °C instead of 27 °C, the slope of the lines decreases, indicating that a temperature of 27 °C is more favourable to both bacteria tested than 17 °C in SPYE. Maximum conductance changes at 17 °C are also lower than those at 27 °C. However, to judge this longer test lengths are needed. In SPYE, Ech 502 generally causes, compared to Pspi 518, strong conductance responses.

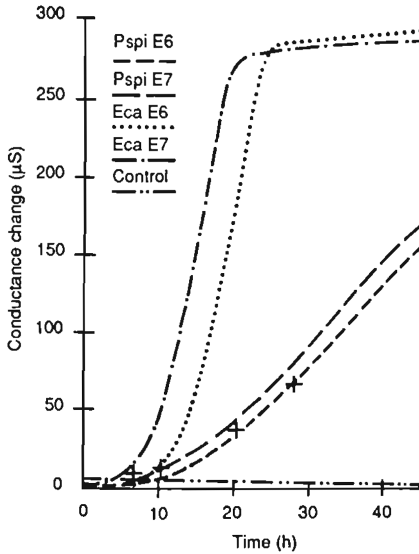

Fig. 1. Conductance changes in SPYE for different cell concentrations of Pspi 518 and Eca 161 in direct conductimetry. E6 and E7 = resp. 10^6 and 10^7 cfu per ml at the start of the test.

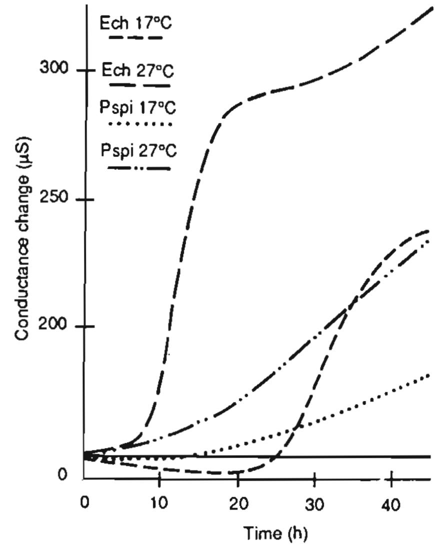

Fig. 2. The effect of temperature on the conductance responses of Ech 502 and Pspi 518 in SPYE at 10^7 cfu/ml in direct conductimetry.

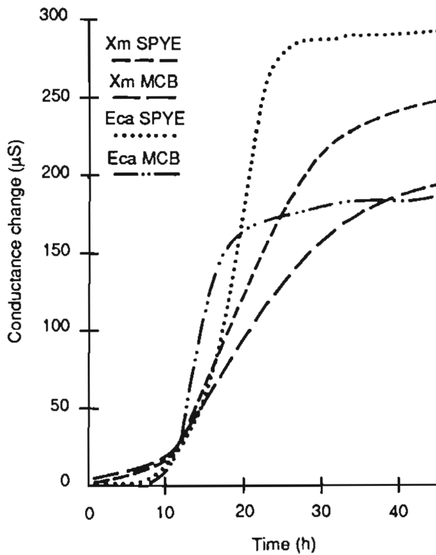

Fig. 3. The effect of different media on conductance changes of Xm 1974 and Eca 161 (10^6 cfu/ml) in direct conductimetry.

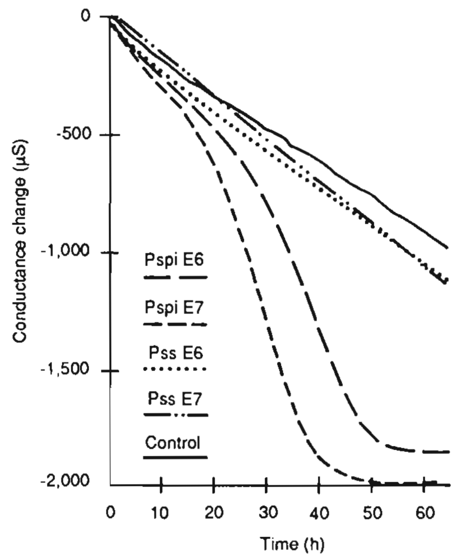

Fig. 4. Utilization of homoserine by Pspi 518 and Pss 1147 in indirect conductimetry.

The effect of different media on conductance responses in direct and indirect conductimetry

Fig. 3 shows that differences between SPYE and MCB can be found in the slopes of the conductance curves and the total conductance change within ca. 48 h. Although generally SPYE gives the best conductance responses, this sometimes depends on the temperature and bacterium tested, e.g. Eca 161 tends to give initially steeper slopes of the conductance curves in MCB than in SPYE.

In indirect conductimetry the utilization of homoserine by Pspi 518 and Pss 1147 was investigated. Fig. 4 shows that Pspi 518 utilized homoserine whereas Pss 1147 did not. Note that conductance changes in the indirect method are negative. Differences between cell concentrations yielded the same shape of conductance. Responses were recorded later in time.

The effect of antibiotics on conductance changes in direct conductimetry

Fig 5. shows the influence of an antibiotic, novobiocin, on conductance responses caused by saprophyte S2C3 isolated from pea seed and Pspi 518. At a concentration of 50 mg/l the growth of S2C3 and Pspi 518 is reduced, which results in lower conductance changes and smaller slopes of the curves. For Pspi 518 the effect of 300 mg/l is less than for S2C3.

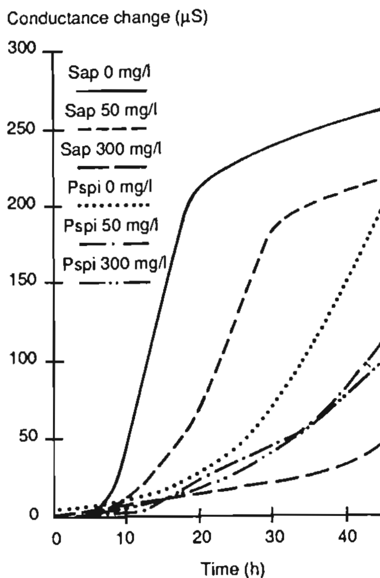

Fig. 5. The effect of novobiocin on conductance responses by saprophyte S2C3 and Pspi 518 at 0, 50 and 300 mg/l (10^6 log cfu/ml).

Direct detection of plant pathogenic bacteria in plant material

Fig. 6 shows the detection of Eca 161 and Ech 502 in potato peel extracts of cv. Bintje, grown on clay or sandy soil, at 17 °C in MEM (direct conductimetry).

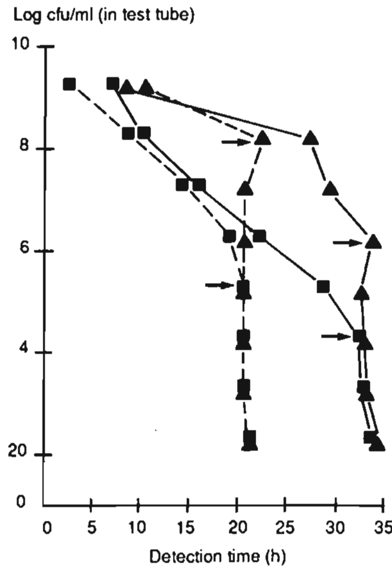

Fig. 6. The relation between the detection time in direct conductimetry and the log number of cfu of Eca 161 (■) and Ech 502 (▲) in peel extracts of potatoes, grown on sandy soil (- - -) or clay (——), at 17 °C. The arrows indicate the level at which the Td becomes inversely proportionate to the log cfu/ml.

The results show that Eca 161 could be detected at lower levels than Ech 502. The sensitivity of the test varied, however, with the origin of the potatoes. The potatoes from the sandy soil yielded lower Td than those from clay, indicating the presence of higher numbers of saprophytes or metabolically more active saprophytes. The sensitivity of detecting Eca 161 and Ech 502 varied from 10^4 to 10^6 cfu/ml, and 10^6-10^9 cfu/ml, respectively.

Discussion and conclusions

In this paper the results of a limited set of strains of plant pathogenic bacteria have been shown. We have also tested more strains of several pathovars of *Xanthomonas campestris*. On the basis of studies with pure cultures, we believe that direct as well as indirect conductimetric assays can be used for studying metabolic activity of plant pathogens e.g.: utilization of specific substrates such as carbohydrates and nitrogen sources, and determination of optimum growth conditions (temperature, media evaluation). With the help of the detection times,

cell concentrations and generation times may be determined. Good prospects exist for detecting *Erwinia* spp. in potato peel extracts. We also have obtained promising results for the detection of *P. s.* pv. *pisi* in pea seeds (B.A. Fraaije, A.A.J.M. Franken, P.S. van der Zouwen, these proceedings). However, media and incubation conditions have to be improved. Possibilities to apply conductimetric assays for the detection of plant pathogenic bacteria in plant material also depend on the generation time of the pathogen and the composition of microbial flora in the plant material. Our aim is to use the detection times, generated by the automated system, for classification of plant material as being "healthy" or as being potentially infested. Presence of the pathogen in seed lots, suspected on the basis of the detection time, can be confirmed with the help of other rapid tests such as serological tests.

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Application of immunofluorescence colony-staining (IFC) for monitoring populations of *Erwinia* spp. on potato tubers, in surface water and in cattle manure slurry

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Abstract

Pour-plating combined with immunofluorescence colony-staining (IFC) offers the advantage over traditional surface plating that it can be performed in a miniaturized routine assay. IFC was used in comparison with other methods to study the population dynamics of *Erwinia carotovora* subsp. *atroseptica* (Eca) and *E. chrysanthemi* (Ech) in cattle manure slurry, surface water and on the tuber surface. The detection level for Eca and Ech in complex environs was 10^2 to 10^3 cells per ml using the semi-selective PT-medium. Isolation from an IFC-positive colony enabled the verification of the detection of the target pathogen and was an efficient tool to obtain strains of cross-reacting bacteria from surface water. Automated counting of IFC-positive and background colonies was simultaneously possible using an image analyzer.

Keywords: *Cross-reaction, Xanthomonas campestris* pv. *pelargonii, X.c.* pv. *begoniae, image analyzer*

Introduction

In traditional plating on the surface of agar media, the detection of low densities of target bacteria in complex environs is often unreliable by the interference of high numbers of background bacteria. The combination of sample pour plating with immunofluorescence colony-staining (IFC) of the target colonies (VAN VUURDE, 1987) forms an alternative to traditional plating.

The aim of this paper is to illustrate that IFC (1) forms a valuable tool for sensitive detection of culturable target bacteria in complex environs, (2) allows verification of positive results by isolation from IFC-positive colonies and effective isolation of cross-reacting bacteria, and (3) can be miniaturized and automated into a routine format. Examples are presented for the detection of Erwinia spp. in complex environs with various methods.

Materials and Methods

Bacterial strains. In experiments with inoculated tubers, inoculation was done with *E. chrysanthemi* (Ech) strain IPO 502 (= PD 226) or *E. carotovora* subsp. *atroseptica* (Eca) strain IPO 161 (= PD 230).

Microbial analysis. ELISA was performed as described by VAN DER WOLF & GUSSENHOVEN (1992). IFC was done in a miniaturized format using 16 mm wells of 24-wells tissue culture plates and isolation from IFC-positive colonies (VAN VUURDE, 1990; VAN VUURDE & ROOZEN, 1990). Plating on crystalviolet pectate medium (CVP) in combination with Ouchterlony double diffusion (ODD) using anti-Ech and anti-Eca sera was done for verification of IFC-results. Similarly, ODD was also used for the characterization of isolates obtained from IFC-positive colonies in PT medium (VAN VUURDE & ROOZEN, 1990). A selection of isolates from IFC-positive colonies was verified with fatty acid profiling by J.D. Janse at the Plant Protection Service (PD) in Wageningen.

Experiments and Results

Survival of Ech on tubers between harvesting and planting. After vacuum infiltration of tubers of cv. Désirée with Ech strain 502 at various densities, tubers were stored at 4 C for 4.5 months and pregerminated for 6 weeks thereafter. Peel (c. 6 cm^2) extract samples of the various treatments (n = 20) were prepared with a Pollähne press for IFC and ELISA at day 1 after inoculation and after 3 and 6 months. The ELISA values were corrected for the OD-value of the control (vacuum infiltration with water); no IFC-positive colonies were found in the control.

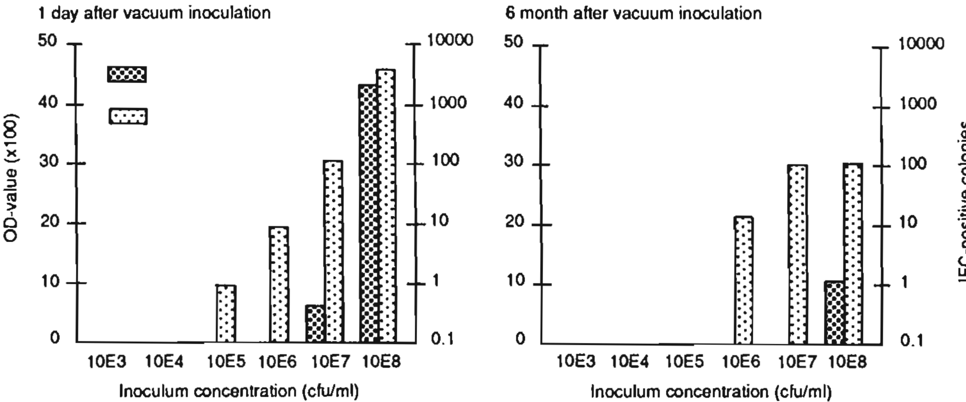

Fig. 1. Population dynamics of Ech on tubers of cv. Désirée after vacuum infiltration of the tubers with Ech at various inoculum densities (n = 20 per treatment).

The histograms in figure 1 show for day 1 that the average number of IFC-positive colonies decreased with the decreasing densities of Ech cfu per ml. The detection level for ELISA was 10^5 cell equivalents per ml and for IFC 10^3 cfu per ml for the peel sample (based on an estimated c. 100 times lower Ech contamination of the peel sample than the Ech density in the inoculum). After 3 months of storage, Ech was only detectable with ELISA in tubers inoculated with 10^8 cfu per ml and with IFC with 10^8 and 10^7 cfu per ml (data not shown). After pregermination, the detectability of Ech with IFC was increased but remained the same with ELISA.

Detection of Erwinia spp. in surface water. Water samples (50 ml) were taken in spring and autumn of 1988 to 1991 at up to 23 locations in the new polders (IJsselmeerpolders). Platings of 0.1 ml and dilutions on CVP and of 0.1 ml in PT for IFC were done for the undiluted sample and after 10 times concentration of the sample by centrifugation. Erwinia-type pits on CVP were verified with ODD, since 1990 IFC-positive colonies were verified after isolation and ODD (VAN VUURDE & DE VRIES, 1992).

Table 1. Detection of Eca and Ech in surface water in the new polders in 1988 to 1991. Positive results were obtained in 1988 and 1989 with CVP/ODD and 1990 and 1991 with CVP/ODD or IFC/ODD.

Year	Number of locations		Number of positive locations			
	Spring	Autumn	Spring		Autumn	
			Eca	Ech	Eca	Ech
1988	5	6	0	0	1	0
1989	6	18	0	0	2	8*
1990	19	23	0	0	2	2*
1991	22	23	0	0	1*	0

* One isolate from one positive location was verified at the PD with fatty acid analysis

Data of table 1 show that no verified positive samples were found for Eca or Ech in the spring. In the autumn Eca or Ech were incidentally present except for Ech in 1989 (8 out of 18 locations positive). IFC was much more efficient in handling large series of samples, but verification was needed because of the presence of cross-reacting bacteria in the sample (table 2). Some ODD-positive isolates of Eca (n=1) and Ech (n=2) in 1990 and 1991 were further studied. Fatty acid profiling and biochemical tests confirmed the preliminary identification of these isolates and showed that the two Ech strains from water belonged to the high temperature preferring biotype 3 isolated only from greenhouse plants in the Netherlands (J.D. JANSE, pers. com.).

Population dynamics of Eca and Ech in cattle manure slurry. Experiment of VAN VUURDE & ROOZEN (1990) showed for Eca or Ech spiked samples of undiluted cattle slurry a detection level for IFC of 10^2 to 10^3 cells per ml. In comparison with other techniques, IFC was c. 10 times more sensitive than plating on CVP and c. 1000 times more sensitive than ELISA. In experiments on the survival of Eca and Ech in cattle slurry (A.G. ELEMA, J.J.E. RASING, N.J.M. ROOZEN & J.W.L. VAN VUURDE, unpublished), tubers inoculated at the hilum end with Eca or Ech were incubated in 10 l containers with cattle manure slurry kept for fermentation at 4, 10, and 15 C (8 replicates per treatment). After ten days the tubers showed spots of decay indicating that bacteria could be released into the slurry. The tubers were removed from the containers and the first sample, 0.1 ml of undiluted slurry, was pour plated for IFC. Thereafter, samples were taken at regular intervals. Data presented in Fig. 2. show that both populations of Eca and Ech decreased in time but that the decline was much faster for Ech than for Eca. After c. 10 weeks, Eca was below the detection level at 15 and 10 C incubation, but still detectable after 14 weeks at 4 C. Ech decreased to a level of a few colonies in 0.1 ml slurry after 4 weeks and was no longer detectable after 7 weeks for all fermentation temperatures.

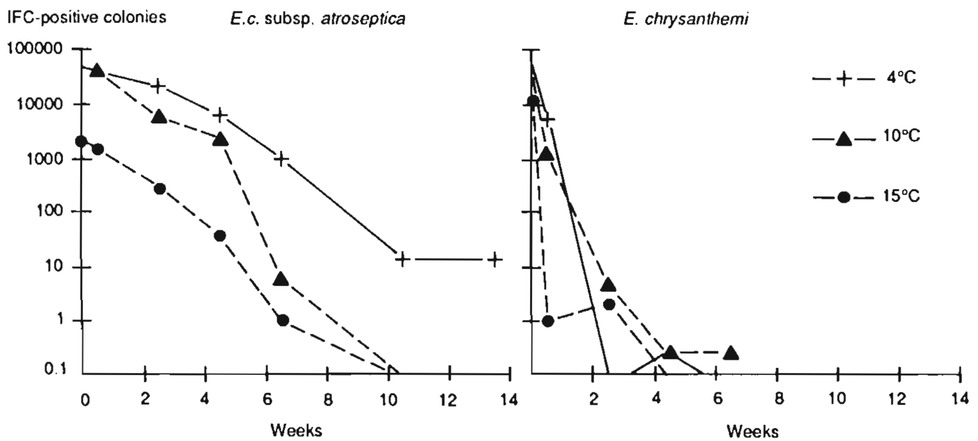

Fig. 2. Population dynamics of *E.c.* subsp. *atroseptica* and *E. chrysanthemi* in cattle slurry after removal of decaying tubers at various fermentation temperatures of the slurry (A.G. ELEMA, J.J.E. RASING, N.J.M. ROOZEN & J.W.L. VAN VUURDE, unpublished).

Isolation of cross-reacting bacteria with IFC. Isolations from IFC-positive colonies were made by puncturing the colony with a fine needle or glass capillary under the fluorescence microscope at 4x magnification. Dilutions of the bacterial smear were streaked on both CVP and trypticase soy agar (TSA). In

the case of absence of erwinia-type pits on CVP, bacteria of colonies on TSA were checked for cross-reactions with various serological tests. Table 2 shows for *Erwinia* and *Xanthomonas* spp. the number of cross-reacting strains obtained by random isolation over the past ten years and by IFC during the last two years. Besides from surface water, cross-reacting strains with anti-Eca and anti-Eca serum were obtained from various substrates (see also VAN DER WOLF *et al.,* these proceedings). The percentage of successful isolation from IFC-positive colonies was determined by various factors (VAN VUURDE, 1990) and was more successful for Eca than for Ech in our experiments.

Table 2. Strains of cross-reacting bacteria of the IPO-DLO collection isolated from different samples, and obtained by random isolation or by isolation of IFC-positive colonies. For further explanation see text.

Serum	Cross-reacting strains		Phenotypes	Source
	Random	IFC		
Anti-Eca	1[2]	7	3	IPO/PAGV
Anti-Ech[1]	1	16	4	IPO/PAGV
Anti-Xcb[3]	1	0	1	IPO/NAKS
Anti-Xcp[4]	0	1	1	NAKS

[1] see Van der Wolf et al., these proceedings; [2] *E.c.* subsp. *carotovora* (serotype 2); [3] Xcb = *X. campestris* pv. *begoniae* (isol. by M. Hooftman); [4] *X.c.* pv. *pelargonii* (isol. by M. Hooftman)

Discussion and Conclusions

Choosing the multi-well tissue culture plates formed the basis for further developments in automation of the assay comparable to ELISA. Automation included the use of multipipets and plate shakers for the various steps of the procedure. Furthermore all steps were performed for a sample in the same well, including the final reading of the stained preparation at 4x objective magnification. Recent experiments demonstrated the possibility to automate the reading of the target and non-target colonies in the wells of series of multi-well plates by linking the epi-fluorescent microscope (Leitz) equipped with a programmable scanning stage to a low lux video camera (HCS PLT450) connected to a Context vision GOP302 image analyzer (J.W.L VAN VUURDE & E.M.J. MEIJER, unpublished).

The presented examples show the potential of IFC as a powerful tool for handling large series of samples for ecological research. Strong points of the technique, besides the possibility to use it in a routine format, are (1) the low detection level and the high range of numbers of (target) colonies which can be detected per preparation (10^4 to 10^5 per 16 mm well) as demonstrated for *Erwinia* spp in cattle slurry, (2) bacteria from IFC-positive colony can be isolated, enabling a check on the reliability of the

technique and providing an efficient tool to obtain strains of cross-reacting bacteria. Characterization of cross-reacting bacteria offers the possibility to select or prepare better quality antisera for IFC and to improve the selectivity of the pour plating medium in order to exclude the growth of cross-reacting bacteria in the medium.
When compared with other methods, IFC was c. 100x more sensitive than ELISA for Ech on potato tubers. For Eca and Ech in cattle slurry, IFC was c. 1,000x more sensitive than ELISA and 10x more sensitive than surface plating on 90 mm pectate medium plates. Research of LEEMAN *et al.* (1991) showed that the detection level of IFC for a fluorescent *Pseudomonas* strain from soil was c. 10^2 and comparable to that of a detection system based on an antibiotic resistant mutant of that strain in combination with a selective medium containing that antibiotic.

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Colonization of potato stems by *Erwinia* spp.; including *in planta* detection in stem sections with immunofluorescence colony-staining (IFC)

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Abstract

Serological and plating methods were used to study the population dynamics of *Erwinia carotovora* subsp. *atroseptica* and *E. chrysanthemi* in stems of potato plants. Numbers of colony forming units (cfu) of the target, determined with immunofluorescence colony-staining (IFC) and with plating on pectate medium, were highest in the basal parts and correlated better with browning of the vascular system for cv. Désirée than for cv. Spunta. Similar results were obtained for the number of immunofluorescence-positive target cells in prints of transversal stem cuts on microscope slides. Handcut stem sections were embedded in agar medium and incubated for *in situ* enrichment prior to IFC. Observation of the sections using 4x and 10x objectives showed colonization of vascular, cortical and epidermal cells and of the intercellular space. Sections which were stored dry for 2 weeks at 4 °C gave similar results as fresh stem coupes for *E.c.* subsp. *atroseptica,* but were less good for *E. chrysanthemi*.

Keywords: *E. carotovora subsp. atroseptica, E. chrysanthemi, population dynamics*

Introduction

Application of immunofluorescence colony-staining (IFC) for ecological studies of bacteria on plant parts was reported by UNDERBERG & VAN VUURDE (1989) and VAN VUURDE (1989). IFC made it possible to obtain an overview of the root colonization pattern of *Erwinia chrysanthemi* (Ech) on potato roots using an epi-fluorescence microscope with a 4x objective (UNDERBERG & VAN VUURDE, 1989). The method is based on the *in situ* enrichment of culturable cells of the target bacterium to microcolonies on roots or root parts embedded in semi-selective agar medium and the use of IFC to distinguish between target and non-target colonies.

The aim of this paper is to evaluate IFC for the detection of target bacteria in stems. The spread of *Erwinia carotovora* subsp. *atroseptica* (Eca) and Ech was studied in potato stems. The possible use IFC to determine infection sites of the target *in situ* in stem sections after microbial enrichment was studied.

Materials and Methods

Plant material. Stems from cv. Spunta and cv. Désirée plants grown from tubers which were vacuum infiltrated with Ech strain IPO 502 (= PD 226), and from plants of the variety Bintje, grown from tubers naturally infected with Eca, were collected in the experimental field at Zeewolde. Plants of cv. Kondor raised in the greenhouse were inoculated at the stem base with Eca strain 1001 (IVIA) 4 weeks after planting and sampled 2 weeks later. Stems segments of c. 2 cm were sampled from the base to the tip.

Microbiological analysis of stem sections and segment extracts. Two handcut (cross-)sections were prepared per segment with a disinfected razorblade. After gently blotting one of the sections between sterile filter-paper, it was placed on a 1.5 mm thick layer of PT-medium (BURR & SCHROTH, 1977) in the well of a 24-wells tissue culture plate and covered with a c. 1.5 mm layer PT medium (pipetted at 45 C on top of the section). The second section was dried between sterile filterpaper and stored with silica gel for 2 weeks at 4 C, before it was embedded in agar. After incubation for 2 days at 27 C, the agar preparation was air dried in the well at 35 C and IFC was done as described below.

Prints of a stem transection were made for each segment by pressing the surface on a microscope slide for immunofluorescence cell-staining (IF).

Extracts of c. 1 cm of the segments were prepared with a Pollähne press and were dilution-plated on crystalviolet pectate medium (CVP) and pour plated in 16 mm wells of a 24-wells tissue culture plate for IFC (VAN VUURDE & ROOZEN, 1990).

Serology. Polyclonal antibodies produced in a rabbit were conjugated with fluorescein iso-thiocyanate (FITC). The conjugates contained 3 mg IgG and were diluted before use 1 : 100 (IFC) or 1 : 300 (IF) with 0.05 M PBS with 0.1 % Tween 20 (PBST). The IF-staining period of the cells was 30 min and of the colonies 16 h at room temperature (VAN VUURDE, 1990). After washing 2 times for 10 min with PBST, the preparations were observed directly in the wells under an epi-fluorescence microscope (490 nm) with objectives 4x (Leitz, NA 0.12,) and 10x (Nikon SLWD, NA 0.21). The presence of the target bacterium on CVP plates with erwinia-type pits was verified by testing a suspension of the colonies of a plate with IF.

Results

Detection of Eca and Ech in stem segments. The various techniques showed a decrease in the number of positive segments from the stem base to the tip (Table 1) for Ech in the stems of plants with symptoms of vacuum infiltrated tubers of cv. Désirée (n = 4) and cv. Spunta (n = 7) and for Eca in the stem segments of plants with symptoms of naturally

infected tubers of cv. Bintje. Stems of plants without symptoms from vacuum infiltrated tubers (n = 4) showed no vascular browning and Ech was only detected in the first 1 to 3 segments of 3 out of 4 stems (data not shown).

Table 1. Colonization of potato stems segments determined with various methods. n.d. = not determined. For further explanation see text.

Stem zone (cm)	No. of Segments	Vascular browning (%)	Positive segments (%)				
			IFC	CVP/IF	IF	Cross-section;IFC	
						Direct	2 wks
Plants with Ech-symptoms; cv. Désirée (n = 4)							
0 - 1	4	100	100	100	100	75	50
4 - 6	4	100	100	100	75	50	25
9 - 11	4	75	75	75	75	50	0
14 - 16	4	25	25	25	25	50	0
19 - 21	4	0	0	0	0	0	0
24 - 26	4	0	0	0	0	0	0
34 - 36	1	0	0	0	0	0	0
Plants with Ech-symptoms; cv. Spunta (n = 7)							
0 - 1	7	86	100	43	29	57	0
4 - 6	7	86	57	14	0	0	0
9 - 11	7	86	57	0	14	43	0
14 - 16	7	86	14	0	14	14	0
19 - 21	7	86	0	29	14	43	0
24 - 26	7	29	43	14	29	29	14
34 - 36	3	0	0	33	0	0	0
Plants with Eca-symptoms; cv. Bintje (n = 3)							
0 - 1	1	100	100	100	n.d.	100	100
4 - 6	3	100	33	67	n.d.	33	33
9 - 11	2	50	100	50	n.d.	100	50
14 - 16	3	67	33	33	n.d.	67	33
19 - 21	3	67	33	33	n.d.	67	0
24 - 26	3	33	33	0	n.d.	67	67
34 - 36	3	33	0	0	n.d.	33	33

The number of positive stem segments was equal (cv. Désirée) or slightly higher (cv.'s Spunta and Bintje) with IFC than with CVP. The enrichment of the target in a section in agar medium followed by IFC was in general more successful than printing on a microscope slide followed by IF. The detection of Eca in the cross-sections after 2 weeks storage was much less affected than that of Ech.

Colonization patterns of stems. Individual stems showed rather large differences in the population dynamics of Eca and Ech from base to tip (Fig. 1). A high correlation was found between a positive result of IFC and vascular browning of the stem segment for cv. Désirée (R = 1.0). However, the correlation was much lower for Ech in cv. Spunta (R = 0.4) and Eca in cv. Bintje (0.5).

In situ detection in stem sections. Figure 2 shows IFC-positive colonies and details of the stem anatomy in sections of cv. Kondor after enrichment and staining with anti-Eca serum. Under the microscope, the yellow autofluorescence and the green FITC-fluorescence could be easily distinguished at the low objective magnifications. Interpretation should be based on series of sections at short intervals. Marking of the cutting direction (by a thin line with an orange-red fluorescent marker pen on one side of the stem) helped to recognize colonization spots which were due to spread of target cfu during the preparation of the segments.

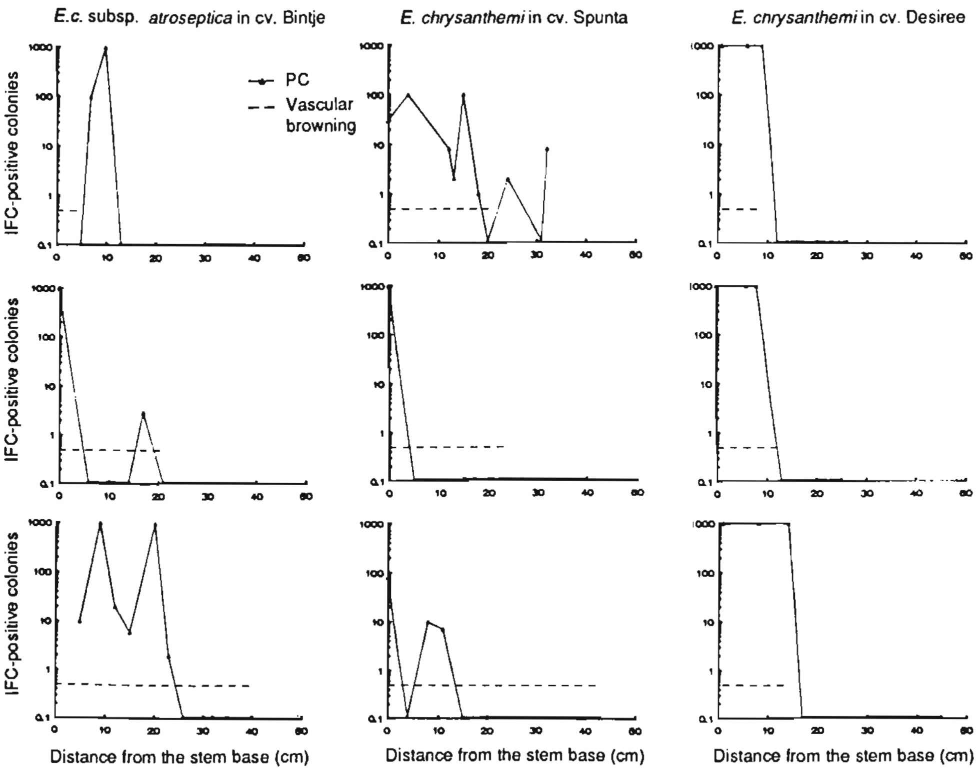

Fig. 1. **Numbers of Ech as determined by IFC for individual stems of inoculated tubers of cv. Désirée and cv. Spunta, and of Eca for stems of naturally infected tubers of cv. Bintje. Plants showed external symptoms, --- indicates vascular browning in the stem.**

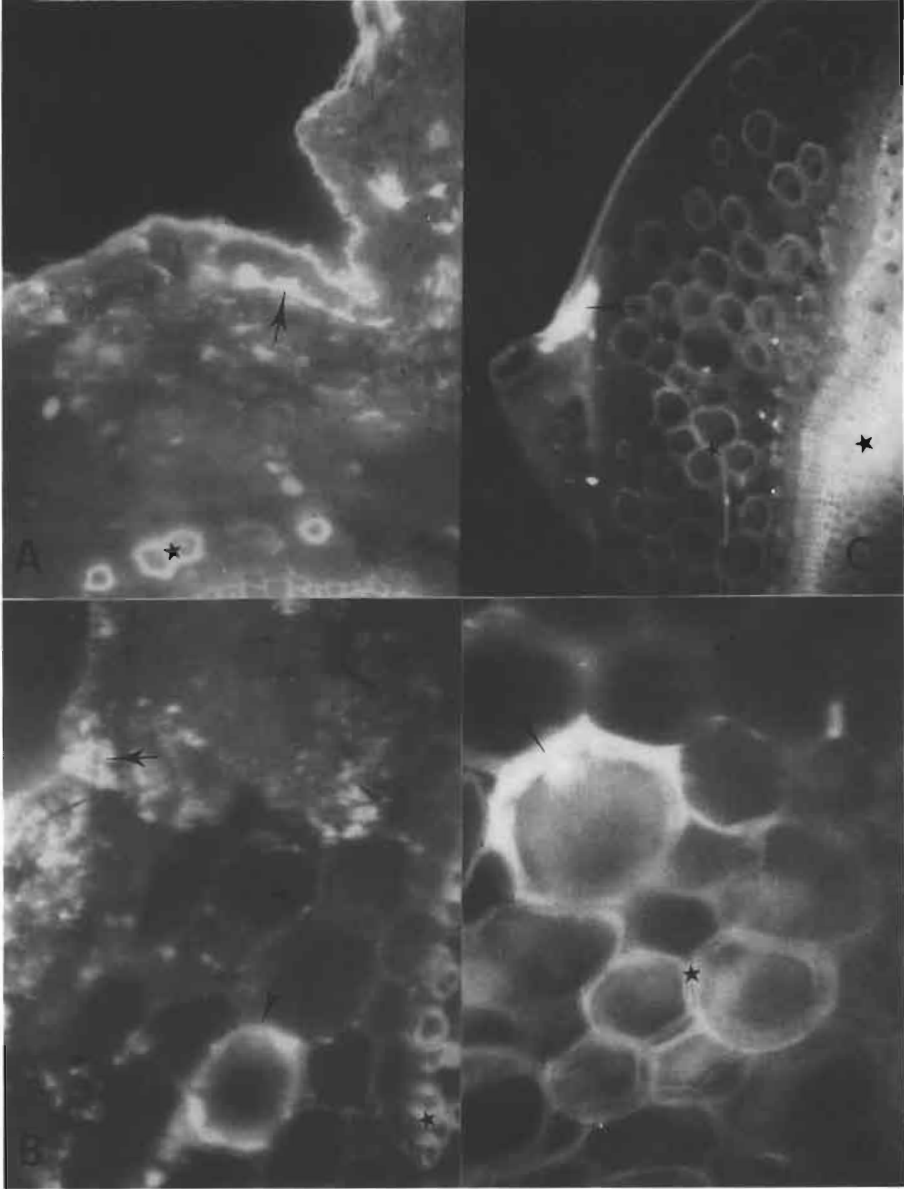

Fig. 2. IFC-positive colonies in cross-sections of Eca-inoculated cv. Kondor stems after enrichment and IFC with FITC-labelled anti-Eca conjugate. Figures 2A and B show colonization (arrows indicate areas of green FITC fluorescence) of cortex and epidermal cells and of intercellular spaces (2B, obj. 10x) at 10 to 15 cm above the inoculation point of the stem. Autofluorescence (stars indicate areas of yellow autofluorescence of lignified cells) of fibers is e.g. visible in figure 2A (obj. 4x). Figures 2C and D show restricted colonization areas and autofluorescence of lignified plant cells in sections closer to the tip; colonization just below the epidermis (2C, obj. 4x), and colonization of intercellular spaces of cortical cells (2D, obj. 10x).

Discussion

Compared with other techniques, the IFC technique showed in our studies good potential for the detection of target bacteria in extracts of stem segments and in stem sections. Critical will be the specificity of the anti-target serum and the specificity of the agar medium. The possibility to isolate from IFC-positive colonies for verification of the target or selective isolation of cross-reacting bacteria (VAN VUURDE, 1990; VAN VUURDE & DE VRIES, these proceedings) forms an important tool to check the reliability of IFC. No evidence was obtained that these organisms interfered in the experiments reported here.

Data from our experiments indicate that vascular browning is positively correlated to infection with Ech (DE VRIES, 1990) and Eca. However, cultivar type and environmental conditions may affect the expression of this symptom and confusion with vascular browning due to fungal infection (*Fusarium* or *Verticillium* spp.) is possible. Target bacteria may also be present at rather large distances above the highest point of the browning or yet below the detection level in stem parts with vascular browning.

Studies with IFC of microbially enriched sections of infected stems will provide information how the infection process in the various stem tissues proceeds with time under various ecological and varietal conditions.

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Effects of cotton stalk briquetting on survival of *Xanthomonas campestris* pv. *malvacearum*

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ABSTRACT

During the briquetting process, pressure and friction heat the feeding stalks and the emerging briquettes temperature was as high has 91°C on the surface and 65°C in the centre. There was also a marked reduction in the moisture content of the briquettes compared to the unbriquetted material.

Briquetting greatly reduced the population of the pathogen, but did not completely eliminate it. The populations on the briquette surface were found to be more adversely affected than those in the centre. However, the bacterial populations consistently decline with holding time irrespective of the treatment.

As the briquette processing does not involve the addition of a binding material, briquettes tend to break with wetting or addition of moisture and remain intact only if kept completely dry.

Keywords: cotton, *X.campestris* pv.*malvacearum*, survival.

INTRODUCTION

Following the introduction of cotton cultivation in the Sudan, it was soon realized that cotton bacterial blight (*Xanthomonas campestris* pv. *malvacearum*) is the main serious disease affecting crop production. The disease is soil borne, in remaining crop debris, and is also seed borne. Annual recurrence of the disease and the enormous crop loss incurred necessitated seeking the adoption of several control measures. One of the first measures taken to reduce crop loss was the enforcement of legislative measures ; the clean-up regulations. The law stipulates the uprooting of cotton stalks after harvest, obliges the farmer to sweep cotton remains and to destroy by fire the stalks and other remains in his tenancy. The legislation was, at that time,

directed towards the control of bacterial blight, leafcurl and the pink bollworm. The latest date fot destroying the old crop and the earliest date for sowing the new one were fixed.

Removal of cotton stalks for domestic use is, until today, a punishable offence. However, changing cropping pattern and rising costs of labour and fuel, justifiably, permitted many illegal practices under the eyes of the law. Legislation has failed to keep-up with the rising needs of the farming community. Since its first day of enforcement the codes of the law remained unchanged. During the last three decades many studies were carried out aiming at the utilization of stalks for a variety of industrial purposes or utilization as energy source, but little study on how to alleviate or modify the legislation without risking the crop were done. The National Energy Administration of the Ministry of Energy and Mining in cooperation with the writers has supported a programme financed by UNDP on effect of cotton waste briquetting on the spread of blackarm. The bacteriological assay was done concurrently by the authors and replicate samples by Drs. Jim Amon and Tim Wood of the Department of Biological Sciences, Wright State University in USA. Results obtained in Sudan are reported herein.

MATERIALS AND METHODS

During the season 1986/87 a very high incidence of bacterial blight was observed on Huda variety in Southern group of the Gezira scheme. Heavily infected cotton stalks with the post-Barakat strain of *X.campestris* pv. *malvacearum* were selected on individual basis and brought down to the ARC Headquarters for further processing. After pulling, the stalks were sun dried for at least two weeks and fed into the mobile Destec briquetting plant through the feeding belt to the chopper where they were shredded into small fragments. A sample from the shredded stalks was drawn to act as a control treatment and the rest was processed into briquettes. Three samples of the briquetted material representing the following treatments were collected.

Treatment number	Machine operating Temperature	Briquette cooling Rate
1	Cool	Normal
2	Normal (hot)	Normal
3	Normal (hot)	Slow

The slow cooling rate is accomplished by immediately packing the briquetted material into sacks and allowed to cool slowly. The originally planned three treatments with variable moisture content were deleted as the briquettes broke on addition of moisture. Instead, however, each of the briquettes samples was separated into two, one representing the outer surface (A) and the other the centre portion (B). This was stimulated by the observed difference in temperature between the two parts when the briquettes come out of the machine.

Moisture content determination

Samples of outer surface and centre samples of briquetted material together with shredded stalks were separately weighed. Samples were then oven dried at 80°C for 24 hrs for moisture determination.

Bacterial counts

All samples were ground into fine powder using Christy Lab Mill (Christy and Norries LTD., Machine Type 8, 8000 RPM). Samples of 1 gm were then suspended in 99ml of sterile distilled water and the flasks were placed in a shaker (Stuart Flask Shaker) for 1 hr, and left ovemight in a refrigerator (4°C), to ensure the release of bacteria from the plant material and prevent bacterial growth. Next day flask shaking was repeated for 4 hrs and aliquots were diluted and plated on X1A agar using overlay method. Plates of the selective medium X1A supplied by Dr.T.Wood were poured 2-3 days before spreading with 0.1ml of the prepared aliquots and dilutions and then incubated for 3-5 days at 25°C. Greenish, mucoid colonies were presumptively identified and counted as *X.campestries* pv. *malvacearum*. This was futher confirmed by flooding plates with iodine solution to test for starch hydrolysis

and by testing randomly selected colonies for pathogenicity. The bacterial numbers were then adjusted according to percent positive isolates.

Pathogenicity tests

Single seedling of Huda variety were raised in small plastic pots 8cm in diameter. Selected colonies were singly suspended in 10cc water in test tubes. The proportion of pathogenic population was determined by injecting the suspensions into the lower epidermis using hypodermic syringes fitted with rubber bungs to the barrel end. Each colony was tested in one leaf. Inoculated plants were kept in a humid chamber in the glasshouse until the time of evaluation.

RESULTS

At the time of briquette harvesting from the machine a marked difference in temperature between the outer and centre portion of briquettes was recorded. The mean temperature of the surface portion was 91°C while that of the centre was 65°C. Furthermore, determination of moisture content has shown substantial difference between briquetted cotton stalk material and shredded stalks and a marginal difference between the surface and centre portions of the briquettes, Table 1.

Table 1 : Moisture content of briquette and shredded cotton stalks

Treatment	**Sample number**				**Mean**
	1	2	3	4	
1 (surface)	2.73*	2.89	2.60	2.67	2.73
1 (centre)	3.56	2.34	2.85	3.36	3.28
2 (surface)	2.26	1.85	3.04	2.27	2.36
2 (centre)	3.36	2.93	2.93	2.36	2.90
4 (shredded)	5.65	8.37	5.95	6.16	6.54

* *Xanthomonas* number x 10^3/gm

Results of the bacterial counts consistently showed higher bacterial populations in the unbriquetted stalks compared to the briquetted material, (Table 2). The lowest bacterial population was always recorded in treatment 2 in which the briquettes were subjected to hot machine temperature and normal cooling rate. The slow cooling rate seems to be more favourable for bacterial survival than either normal cooling rate or low machine temperature at the time of briquetting. This is indicated by the high bacterial populations in treatment 3 especially in the first two counts.

Data presented in Table 2 refer to the corrected counts of the suspected *Xanthomonas* colonies on the basis of the basis of the positive isolates in the pathogenicity tests. Pathogenic isolates produce typical water-soaking symptoms within 3-5 days.

Table 2 : Bacterial population in briquetted and unbriquetted cotton stalks

Treatment	**Description***		**Date**	
		16/7	01/8	15/8
1	Cool/normal	7.66**	10.23	11.87
2	Hot/normal	4.71	1.61	0.74
3	Hot/slow	37.04	34.6	6.55
4	Unbriquetted	97.61	71.47	60.17

* Refers to machine temperature/cooling rate of briquettes.
** *Xanthomonas* number x 10^3/gm.

The results summarized in Fig.1 showed that the bacterial populations in the briquette surface were consistently less than those in the centre. They also revealed a marked decline in bacterial population of all treatments with storage time. Similar trend was also found in the per cent pathogenic isolates of the suspected *Xanthomonas* colonies. In the first pathogenicity test 47% of the suspected *Xanthomonas* colo-

nies from the briquetted material and 81.3% from the shredded stalks proved to be pathogenic. This ratio dropped greatly in the last count to 18.7% for the former and 56.3% for the latter.

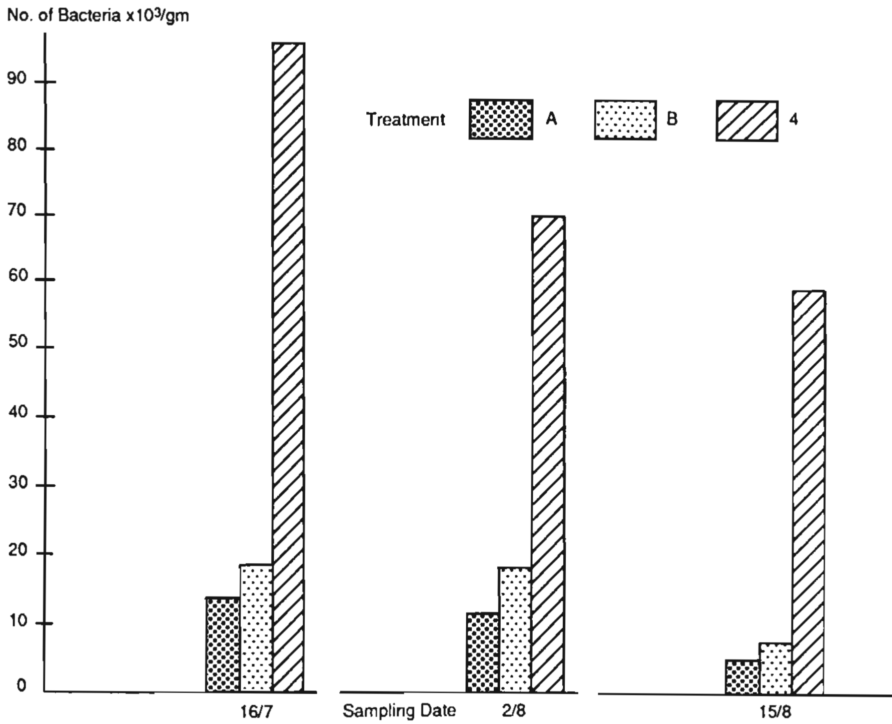

Fig.1. Effect of holding time on bacterial population of X. *campestris* pv. *malvacearum*

DISCUSSION

The results of this study revealed that the bacteria can survive and stand in plant tissues adverse conditions of high temperature and low humidity. The protective role of plant tissue for bacterial survival is now a well known fact. As early as 1930 MASSEY reported that the cotton bacterial blight organism can withstand dessication and heat when he proved the presence of the living causal agents in crop remains after 5 months. The organism can stand a temperature of 72°C for a period of 36 hrs. It is expected that dry woody tissues may retain the organism for a fairly long period

of time. Our results clearly indicate that in woody tissues the organism can withstand temperatures as high as 91°C recorded on the surface of the briquettes.

BRINKERHOEF and FINK (1964) have found that cotton debris burried on moist soils lost their infectivity in 40-107 days depending on the extent of microbial decomposition of the debris. Furthermore in 1961, ARNOLD *et al.*, in Lake Province of Tanzania showed that the trash on the soil surface retained its infectivity longer than burried trash. PARKER (1957) and BROWN (1980) showed that infectivity and survival of the pathogen in debris are adversily affected by rainfall and wettness.

The results of this investigation show that briquetting does not eliminate the bacteria, although considerable loss in population was observed, and therefore the briquettes can provide a source of inoculum to the next crop if not properly stored and used with precautions. It is recommended that the briquettes should be stored indoors as exposure to rain breaks the briquettes into shredded fragments which increase the risk of contamination to adjacent field plots. It is also recommended to use modern methods for collection of stalks and transportation without interfering with other clean up practices followed and also the briquetting machine should be placed away from the field to avoid the risk of contamination during processing.

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Pseudomonas canker of stone fruit trees: a probable predisposing effect of coarse soils related to the twig water content in winter

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Abstract

Bacterial canker generally develops on stone fruit trees in winter and is more severe on coarse soils. In addition to the summer water stress previously studied, an increase of the twig water content in winter, related to the coarse texture of these soils, was noticed. It was assumed to also predispose the trees by favouring the development of the bacteria in the tissues. Field and laboratory studies support this hypothesis.

Key Words: bacterial canker, Pseudomonas, Prunus, environmental conditions, water soaking, water content, soil texture.

1. INTRODUCTION

The symptoms of bacterial canker, induced on stone fruit trees by *Pseudomonas syringae* (pathovars differ according to the fruit tree species), are generally observed in spring. In fact, cold winter temperatures cause the development of tissue infections (KLEMENT et al., 1974; VIGOUROUX, 1974; WEAVER, 1978). This phenomenon has been partly attributed to the effect of negative temperatures which cause water-soaking and subsequent diffusion of bacteria in the cortical tissues (VIGOUROUX, 1991a).

Moreover, bacterial diseases are often more serious in orchards on coarse acid soils (WEAVER & WEHUNT, 1975; VIGOUROUX & HUGUET, 1980). We previously correlated this phenomenon with the occurrence of summer water stress which seems to upset calcic nutrition in these trees (VIGOUROUX & BUSSI, 1989).

However, some experimental results and field observations suggest an other predisposing effect of coarse soils related to the twig water content in winter. This hypothesis is based on the fact that bacterial diffusion in cortical tissues, induced by frost-related water-soaking, is enhanced by increased tissue water content (VIGOUROUX, 1991b). Moreover, YOUNG (1974) showed that water saturation of the intercellular spaces promoted bacterial multiplication in these areas. We thus compared the winter water content in twig tissues of peach trees grown on fine and coarse soils, and also the extent of bacterial infection in peach twigs with different water contents.

2. MATERIALS AND METHODS

2.1 Comparison of water content in the field

Lands in the mid-Rhone Valley are composed of recent fine alluvial deposits alongside sandy-rocky areas formed on ancient glacial terraces. On these two types of soil, observations were carried out on plots with similar varieties of peach trees, including the 'Spring Lady' cultivar grafted on GF 305 and the 'O. Henry' cultivar also grafted on GF 305, the trees were 7 and 8-years-old respectively. 3 to 4 one-year-old twigs 35-45 cm long were harvested from the mid-section of each tree at 15 day intervals from late November to late February for the first cultivar, and from early January to late February for the second. Thus, at each date 15 twigs were removed 4 separate times at each plot. The twigs were enclosed in plastic bags, taken immediately to the laboratory and weighed. They were then oven-dried at 80°C for 48 h to determine their water content.

At the same time, we monitored the soil matrix potential at 30 cm depth using a Watermark probe (Irromater Co. patent) to assess humidity conditions in the two types of soil. Oxygen content could not be measured.

2.2 Comparison of bacterial infection development

These experiments were carried out using twigs collected in a plot of 4-year-old peach trees with the 'Aline' cultivar grafted on GF 305 grown on glacial terrace soil. 4-5 twigs were removed from the mid-section of each tree in late February, thus forming a group of 110 similar one year twigs 40 cm long. In the laboratory, these twigs were divided into two identical groups (groups A and B). Twigs of group A were spread out and left on a laboratory table at room temperature (19-20°C), whereas group B twigs were enclosed in a plastic bag which was then placed amidst group A on the same table. After 4 h, 30 twigs were taken from each group, weighed and oven-dried at 80°C to compare the water loss in both groups. The 25 remaining twigs in each group were artificially inoculated at 2-3 internodes with the bacterium *Ps.s.,* pv. *persicae* (LUISETTI et al.) YOUNG et al., , strain S32* . The inoculation technique involved using the bacterial penetration and diffusion phenomenon associated with frost-related water-soaking (VIGOUROUX, 1991). Hence, 63 inoculations were carried out on slightly dehydrated twigs and 66 on fresher twigs upon removal from 3.5 h storage in a chamber at -6°C.

All twigs were again enclosed in a plastic bag and transferred to a chamber maintained at +10°C. After one week, twigs were examined at regular intervals to detect any glassy, brownish spots indicating success of the artificial bacterial inoculations.

3. RESULTS

3.1 Comparison of water content in the field

Water content variations of both studied cultivars on fine and coarse soils are shown on the graphs in Fig. 1. The water content was found to be consistently higher on coarse soils for both cultivars. Other data that we collected in different situations were in agreement.

*Strain S32, kindly donated by our colleagues J. LUISETTI and J.C. GAIGNARD, at the INRA Phytobacteriology Laboratory in Angers, France.

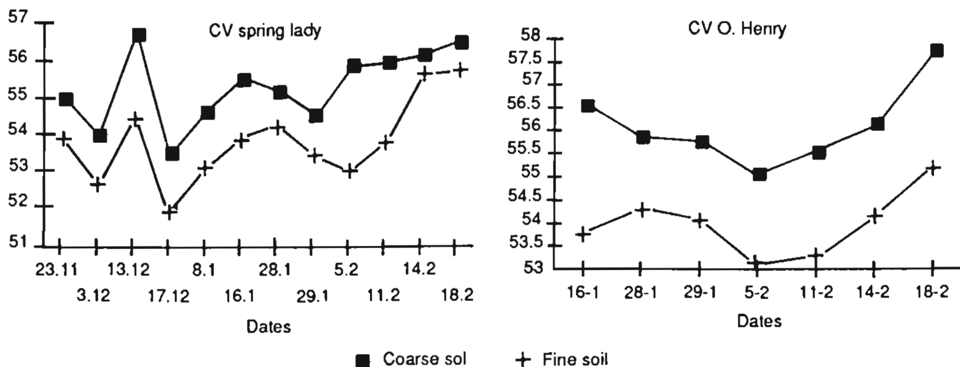

Fig.1. Twig water content and soil texture

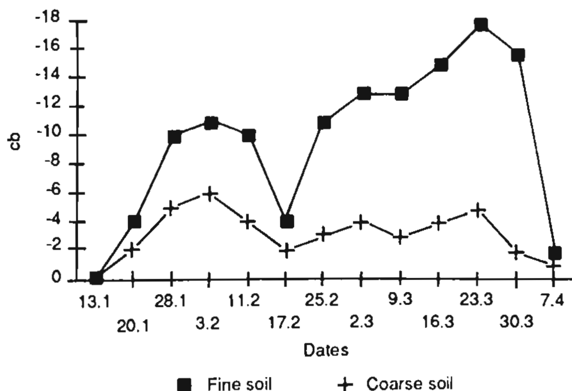

Fig.2. Winter soil matrix potential
in a coarse and a fine soil

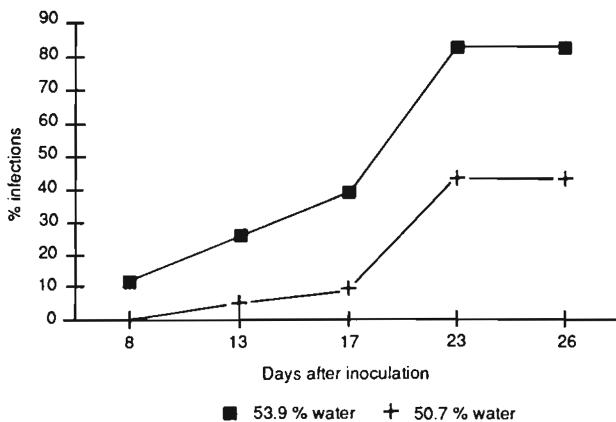

Fig.3. Effect of twig water content
on success of inoculations

3.2 Comparison of soil matrix potentials

Soil matrix potentials measured in fine and coarse soils in winter are shown on the graph in Fig. 2. The potential was always higher (i.e. closer to 0) in coarse soils, except after rainfall when the measurements were almost identical.

3.3 Comparison of bacterial infection development

The water contents of group A and B twigs were 50.7% and 53.9% respectively.
The rates of appearance of bacterial infections for both twig groups are compared on the graph in Fig. 3. Bacteria appeared sooner and inoculation success was much higher for the more hydrated group B twigs.

4. DISCUSSION AND CONCLUSION

The water content of peach twigs in winter was higher when the trees were grown on coarse soils and the observed differences in water content were comparable to those previously found to be responsible for substantial differences in bacterial diffusion during thaws (VIGOUROUX, 1991a). The bacterial infections developped better in the more hydrated twigs. These infections were obtained by a freezing - thawing cycle as it occurs in orchards during winter and the twig water contents were normal for both batches of twigs. So the experimental conditions were completely realistic. This suggests that, in addition to other mechanisms, coarse soils have a predisposing effect on peach trees by increasing the tissue water content in winter.

The soil matrix potential differences observed in the present study could explain this increase. These differences are in agreement with results of overall soil physics investigations comparing water retention in fine and coarse soils (YONG & WARKENTIN, 1975). It is also known that roots have a weak absorbing capacity at low soil temperatures, which varied from +9°C to +4°C in our soils. In these conditions, low soil suction could even further limit the quantity of water absorbed.
Another crucial factor could be the lower oxygen levels in fine soils that also reduce the absorbing capacity of roots in comparison to that of roots in more aerated coarse soils. Besides, MATTHEE & DAINES (1968) compared soils that were similar to those in our study and demonstrated an effect of high oxygenation in coarse soils, at saturating moisture levels, causing water-soaking of foliage and high *Xanthomonas campestris* pv. *pruni* infections in growing peach trees. This water-soaking phenomenon is clearly quite similar to frost-related water-soaking which occurred in winter dormant trees in the present study.
The fact that we obtained similar partial results in apricot (VIGOUROUX, 1989) suggests that this water-soaking phenomenon is widespread in stone fruit trees. Finally, these data should introduce an other aspect to the soil-plant-bacteria relationship which seems to involve a host of factors at different seasons.

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The development of black rot in cabbage as a result of differences in guttation between cultivars, and the relation of guttation to infectiousness

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ABSTRACT

Hydathode infection is a natural entrance of the black rot pathogen *Xanthomonas campestris* pv. *campestris* into cabbage plants. This way of infection requires a path of fluid as it is achieved by guttation. Variation in guttation among cultivars of white cabbage appeared to exist. The black rot susceptible cultivar Perfect Ball guttated more than the less susceptible cultivars Bartolo and Erdeno. A trend was obtained that lesion development was positively correlated with guttation. Estimates of the infectiousness of guttation fluid were made using an efficient technique for assessing bacterial densities. Guttation fluid of root inoculated plants contained the pathogen in 0.8% of the guttation droplets up to about 10^8 cfu ml^{-1}.

INTRODUCTION

Black rot, caused by *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson 1939 is the most threatening bacterial disease in cabbage crops (WILLIAMS, 1980). Many efforts are being made to reduce the impact of this disease such as breeding for resistance and sanitary measurements among which seed health testing. It is generally accepted that relatively high temperatures and high humidity favour the disease. How these driving variables are quantitatively related to disease development is scarcely known.

The pathogen usually invades the plant through the hydathodes at the margins of the leaves. The guttation droplets formed at those hydathodes, as a result of high root pressure and a low evapotranspiration from the leaf, seem to be crucial in this process. As soon as guttation droplets are present, a path of liquid exists which enables the bacterium to invade (COOK et al., 1952). If guttation does not occur, infection will not take place and no disease will develop. Differences among cultivars with respect to degree and duration of guttation should therefore lead to differences in disease development. This paper reports on experiments carried out to study a possible cultivar dependency of guttation and subsequent lesion development. A part of the experiments was carried out with root inoculated plants in order to make estimates on the infectiousness of guttation fluid from these plants.

MATERIALS AND METHODS

Bacterial strain. Two days old cultures of *Xanthomonas campestris* pv. *campestris (Xcc)* strain LUF 107 (= PD 714[1]) grown at 26°C on yeast peptone glucose agar (YPGA) were used in the experiments. Bacterial suspensions were made in phosphate buffered saline (KH_2PO_4 0.1%, $Na_2HPO_4.12H_2O$ 1.45%, NaCl 0.8%).

Host plants. White cabbage plants, *Brassica oleracea*, cvs. Perfect Ball (Nickerson-Zwaan), Bartolo (Bejo) and Erdeno (Zaadunie) were used in the three to five leaves stage. Plants were grown in standard potting soil (Trio-17) and incubated in a walk-in climate room with a day(16h)/night(8h) temperature regime of 22/14°C and a relative humidity (RH) of about 80 %. Illumination was about 8000 lux. To obtain a condition with 100% RH the plants were placed in a polyethylene cage (1.8 x 0.7 x 0.4m) which was opened when a situation was required with 80 % RH.

Inoculations with Xanthomonas campestris pv. campestris.

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[1] PD = Culture Collection of the Plant Protection Service, Wageningen, The Netherlands

- Inoculation of the plant roots. After damaging the roots in the plant pot with a scalpel, 10 ml of a *Xcc* suspension (10^8 cells ml^{-1}) was poured into the soil using a funnel to avoid contamination of the soil surface.
- Inoculation of the guttation droplets. A bacterial suspension with 3×10^8 cells ml^{-1} was atomized over the plants with an ULVA ultra low volume sprayer (Micron Sprayers Ltd). It was essential to avoid loss of guttation droplets by run off.

Experimental design.

- Experiment 1. *Cultivar dependent guttation and infectiousness.* Sixty four cabbage plants (cvs. Perfect Ball and Bartolo) were used in the experiment. Of each cultivar 26 plants were root inoculated with *Xcc*; six plants were not inoculated and used as a control to test if root inoculation had an effect on the guttation. The plants were incubated at 100% RH. The guttation index (see below) was assessed during a period of 21 days. The assessment took place as frequently as possible, but not every day. Guttation droplets from half of the root inoculated plants were examined to determine the presence and density of *Xcc* in the droplets to find a measure of the infectiousness of the guttation fluid of the plants (experiment 1A). This experiment was repeated with a day/night regime of the relative humidity of 80%/100% (experiment 1B).
- Experiment 2. *Guttation and lesion development.*

Fifteen cabbage plants per cultivar (cvs. Perfect Ball, Bartolo and Erdeno) were incubated during two days at 100% RH. After formation of guttation droplets, the number of droplets per leaf (the two last, fully developed leaves per plant) were counted and inoculated by atomizing *Xcc* over the guttating plants. The air humidity was subsequently reduced to 80% in order to stimulate the withdrawal of the guttation droplets into the leaves. The plants were subjected to the normal climate room regime (25 °C, 85% RH). The final assessment of the number of lesions per leaf was made after 23 days.

Guttation index. The degree of guttation of the leaves was indexed by means of an index with three levels. Level 0: no guttation at all; level 1: not all the leaves of the plant bear guttation droplets, and only a few droplets per leaf if guttation occurs; level 2: all the leaves of the plants guttate abundantly. Per

cultivar, the guttation index for each day of observation per cultivar was calculated according the following formula:

guttation index $I_t = N(2)_t*2 + N(1)_t*1$

with t = day of observation,

$N(2)$ = number of plants guttating according level 2,

$N(1)$ = number of plants guttating according level 1.

Estimation of Xcc density in guttation fluid. For the estimation of the bacterial density in guttation fluid, droplets of guttation fluid were removed from the leaf by sucking the fluid into a strip of sterile filter paper (0.3 x 7 cm). As a rule each droplet was examined separately, but if the droplets were too small, several droplets were combined. As soon as disease symptoms appeared, the collection of guttation droplets was terminated. The strip was streaked over a distance of 9 cm onto a wide (15 cm) YPG-agar plate. The plate was incubated for 44 hours at 27°C. The outgrowth on the plate was indexed according the following index. The density of colonies in each 3 cm of the 9 cm streak distance was classified from 0 to 3; 0: no colonies; 1: few separate colonies; 2: many separate colonies; 3: dense confluent growth of colonies (Figure 1). The levels of bacterial growth in the 3 cm parts of the streak on the plate are added numerically. The maximum index for the growth of the bacterium is therefore 9. The correlation of the growth index (GI) upon bacterial density (D) is given by $GI = 0.34 + 9.3/\{1 + \exp[-1.08*(D-7.74)]\}$, $R^2 = 94.1$ (See Figure 2).

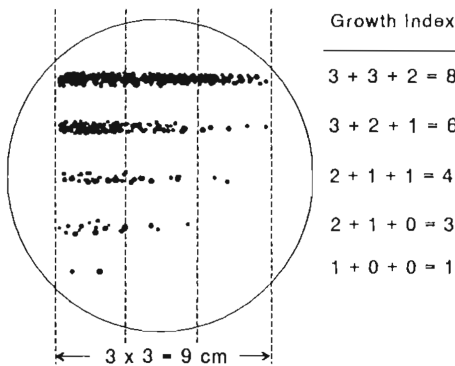

Figure 1. Growth Index for estimation of bacterial density in guttation fluid using the streak method

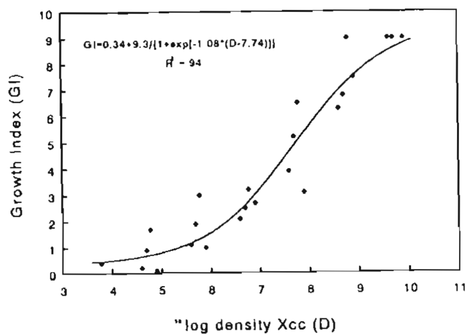

Figure 2. Correlation of Growth Index upon ^{10}log density of *Xanthomonas campestris* pv. *campestris*

Statistical evaluation of the data. The data were statistically analyzed by regression analysis and tested for significance using the t-distribution.

RESULTS

Cultivar dependent guttation and infectiousness. Root inoculation had no significant effect on the guttation of the plants. Therefore all guttation assessments were used to evaluate the guttation in relation to the cultivars used. Marked differences were found in relation to the level of guttation of cabbage cultivars. The differences between cultivars were most prominent at the beginning of the experiment. A comparison of guttation indexes on succesive observation dates showed that the differences lasted for the whole period of the experiment (Figures 3A and 3B). During the guttation experiments 1A and 1B, guttation droplets were collected and plated to estimate the density of *Xcc* in the fluid using the Growth Index (Figure 1). In experiment 1A (continuous 100% RH) *Xcc* was recovered from guttation fluid in only one guttation droplet on day 21 from the 84 droplets that could be examined. In experiment 1B 1277 droplets were examined over a period of 21 days, and 0.8% of the droplets contained *Xcc* ranging in densities from

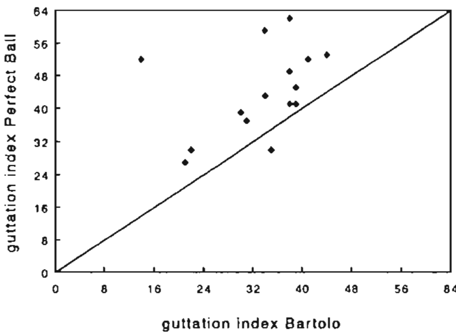

Figure 3A. Guttation index of white cabbage cv. Perfect Ball as compared with the guttation index of cv. Bartolo at 100% Relative Humidity. Entries are guttation index per observation date

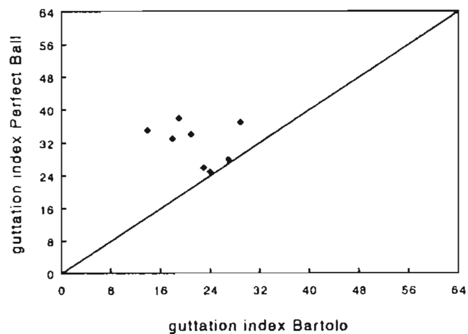

Figure 3B. Guttation index of white cabbage cv. Perfect Ball as compared with the guttation index of cv. Bartolo at 80% (day) 100% (night) Relative Humidity. Entries are guttation index per observation date

$1.6 * 10^5$ to $1.6 * 10^8$, mainly in cultivar Perfect Ball. Cultivar Bartolo produced small guttation droplets so that in many cases not enough fluid could be collected.

Relation guttation and lesion development. In this experiment the number of guttation droplets present at the margins of the leaves increased over time from the onset of the experiment (plants at 100% RH) with a maximum for the cultivars Perfect Ball, Bartolo and Erdeno on days 5, 7 and 4, with an average of 7.4, 4.2, 6.6 droplets per leaf respectively. The plants were inoculated on day 7. At that time the Perfect Ball plants had the highest level of guttation (an average of 6.5 droplets per leaf); the cultivars Bartolo and Erdeno had 4.2 and 3.0 droplets per leaf, respectively. The variation in the number of droplets per leaf was large, and there was no statistically significant difference between the number of droplets per leaf per cultivar at the time of inoculation. Large variation was also found in the numbers of lesions per leaf per cultivar and no significant correlation was found between the number of droplets per leaf and the number of lesions on the same leaf. In an attempt to reduce the variance the results of the 15 plants were randomly grouped in three subunits of five plants, and the average number of guttation droplets and subsequent numbers of lesions were calculated. These data (Figure 4) show a tendency that more guttation per leaf results in more lesions. However, this correlation has a correlation coefficient r of 0.62 and is significant at a probability level between 0.05 and 0.1.

DISCUSSION

Cultivar dependent guttation. Under artificial conditions in climate rooms evidence is obtained that cabbage cultivars differ in their abilities to guttate. The difference is based both on guttation rate and amount of guttation. In 1991, field observations on guttation of the cultivars showed only on two of eleven observation dates a small difference between the cultivars. On most of the observation dates there was either no guttation or abundant guttation in all three cultivars. Because it was impossible to observe the guttation on rainy

days or days with strong winds a complete picture could not be obtained. Field and indoor results lead to the hypothesis that differences between cultivars in respect to guttation follow the pattern as given in Figure 5. This indicates that differences in guttation among cultivars depend on how favourable the guttation conditions are. The relation between guttation and lesion development under field conditions seems to be difficult to study because the spread of the bacterium will be most intensive under conditions of rain and wind (KUAN et al., 1986). It is just in these situations that guttation is impossible to assess.

Infectiousness of guttating plants. The results obtained in these experiments confirm earlier results (RUISSEN et al., 1989) that cabbage plants can be infectious before symptoms become apparent. Again it is shown that differences in the relative humidity is of importance. It is, however, not the guttation fluid only that is responsible for the infectiousness of cabbage plants. As soon as lesions appear on the leaves, *Xcc* bacteria could be washed off from the leaves (up to about 10^4 cfu per cm^2 lesion surface). No data were obtained on the length of the infectious period.

Relation guttation and lesion development. The measurement of the number of droplets at the time of inoculation needs some further discussion. Does this measurement correctly estimate the number of functional entrances for

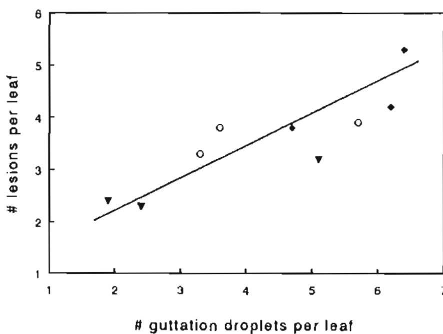

Figure 4. Relation between number of black rot lesions per leaf and number of guttation droplets per leaf. ▼ = cv. Erdeno, O = cv. Perfect Ball, ◆ = cv. Bartolo

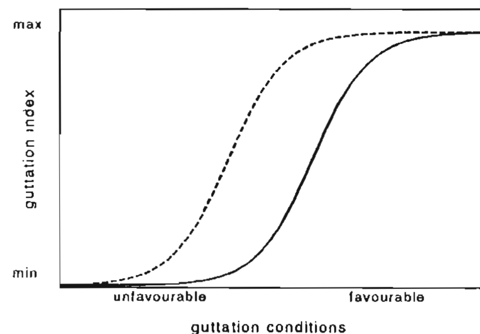

Figure 5. Hypothetical relation of guttation upon external conditions for two different cabbage cultivars. Solid line : cultivar I, broken line cultivar II

the bacterium? The invasion of the bacterium into the plant tissue requires a continuous path of liquid from the inner tissues of the plant to the apertures in the epidermis (COOK et al., 1952). This point was confirmed by the additional experiment with spray inoculation of guttating and non-guttating plants. Only plants that guttated gave black rot symptoms. The assessment the level of guttation is not enough. Situations can occur where a guttation droplet becomes too heavy and falls. Notwithstanding the loss of the droplet, the liquid path is still present, and bacteria can still invade the plant. It might also happen during inoculation that a guttation droplet increases too much in size, and falls off. Then no bacteria are present at the hydathode aperture, and no inoculum is withdrawn into the inner tissues of the plant when evapotranspiration from the leaf increases.

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Scanning electron microscopy of *Pseudomonas viridiflava* colonizing kiwifruit leaves

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Abstract

The colonization of the leaf surfaces of kiwifruit *(Actinidia deliciosa)* was followed after the artificial contamination of young potted plants with *Pseudomonas viridiflava*. After a decrease, at the beginning, bacteria colonized mainly in the hollows, on and within stomata.

Keywords: Scanning electron microscopy, *Pseudomonas viridiflava*, kiwifruit, phylloplane.

Introduction

Pseudomonas viridiflava (Burkholder) Dowson is the causal agent of bacterial blight of kiwifruit [*Actinidia deliciosa* (A. Chev.) Liang *et* Ferguson].

BALESTRA (1991) found that the pathogen is present on symptomless leaves of kiwifruit and that the values of its epiphytic population fluctuate during the different months, depending mainly on climatic conditions. Moreover, bacteria sprayed on kiwifruit leaves were able to colonize the phylloplane (YOUNG *et al.*, 1988).

The aim of this work was to monitor, by means of a Scanning Electron Microscope (SEM), the epiphytic colonization of leaves by *P. viridiflava*.

Materials and methods

A bacterial suspensions of 10^7 cfu/ml was sprayed on the leaf surface of young potted plants. Up to 20 days after contamination several leaves were taken at random and processed for observation at SEM (ROOS & HATTINGH, 1983).

Results

Very few bacterial cells were observed on the leaves one hour after spraying. After 24 h the number of bacteria began to increase and bacteria were seen as microcolonies mainly in the hollows of the leaf surface (Fig. 1). After 48 h bacteria colonized all leaf surfaces and were also seen on and

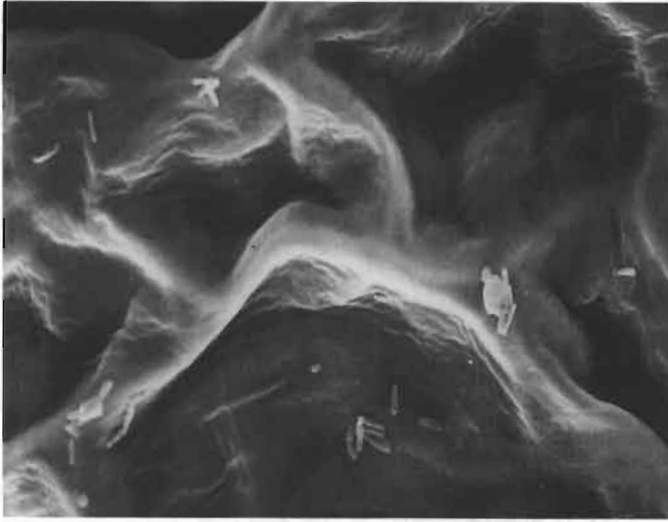

Fig. 1. *Pseudomonas viridiflava* cells on the leaf surface 24 h after spraying (x1,890).

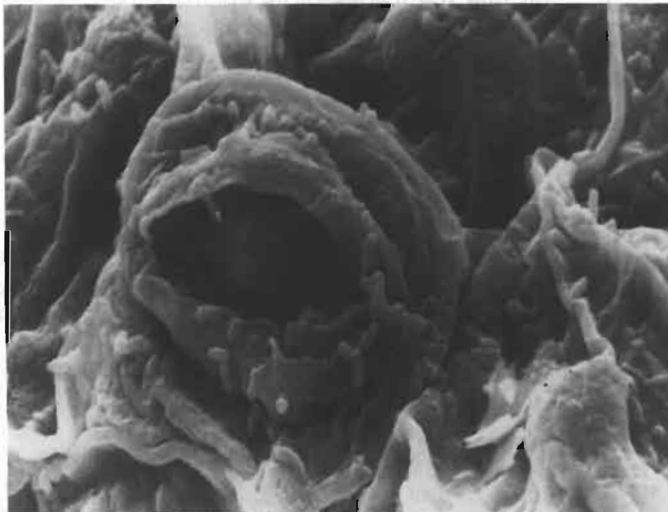

Fig. 2. Leaf surface colonized by *Pseudomonas viridiflava* 48 h after spraying (x2,700).

within stomata (Fig. 2). The bacteria were observed in larger numbers on the lower rather than on the upper leaf surface. After 5 days the number of visible bacterial cells diminished, reaching values which thereafter remained steady throughout the experiment.

Discussion

The above demonstrated that *P. viridiflava* is able to survive on and to colonize the kiwifruit phylloplane.

Protected sites on leaf surface, as hollows or stomata, let the bacteria survive on kiwifruit leaves. In fact, after an initial decrease of their number, bacterial populations sprayed on the leaves increased, reaching values of the order of 10^4 cfu/cm^2 of the leaf (FABI, 1991). In this way an important source of readily available inoculum on kiwifruit phylloplane has been established.

Besides, since some isolates of *P. viridiflava* are active in ice nucleation, its presence on the phylloplane may increase the sensitivity of the plants to frost injury (VARVARO, 1992).

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Pea seed contamination by *Pseudomonas syringae* pv. *pisi:* description and consequences

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SUMMARY

Pea blight, caused by *Pseudomonas syringae* pv. *pisi* (PSP), is seed transmitted.

Bacterial distribution within seed and within seed lot was investigated. By separated analyses of coats and cotyledons, it was found that, for the seed lot tested, PSP was mainly associated with coats, either on the seed, or on the internal face of coats (between coats and cotyledons). Seed by seed analysis of 4 different lots has shown that PSP was more frequently detected in seeds showing lesions and that one seed could shelter as many as 10^6 viable cells.

To study the effect of the amount of bacteria carried by seed on subsequent epiphytic populations and disease incidence, seeds were inoculated with different concentrations of a marked strain, using vacuum-infiltration. High correlation was found between the amount of bacteria on seeds and population levels detected 8 days after sowing. The correlation was also high between the amount of bacteria per seed and symptoms that appeared 15 days after sowing.

The occurrence of PSP on plants grown from naturally infected seeds was studied in comparison to seeds disinfected with streptomycin. The random distribution of the pathogen on seeds provide foci of primary inoculum. Less foci were observed 42 days after sowing in the disinfected plot, but 88 days after sowing, the rapid epiphytic spread of PSP has invaded a high proportion of plants in the whole trial. No symptoms at all were observed during all the experiment time.

The conducted experiments support the fact that the role of seed infection is crucial in pea blight epidemiology.

KEY WORDS : *Pseudomonas syringae* pv. *pisi*, pea, seed infection, contamination description.

INTRODUCTION

Pseudomonas syringae pv. *pisi* (PSP), the causal agent of pea bacterial blight, is seed-borne. Infected seeds are the main source of primary inoculum in field. Bacterial distribution at the seed and the seed lot levels is unknown, and so

was investigated. This knowledge would help us to improve the reliability of seed lot routine analyses. We also studied the relation between seed infection and subsequent disease outbreaks. The development of the pathogen from a natural seed infection was finally described, in comparison to disinfected seeds.

MATERIALS AND METHODS

Identification

When naturally infected seed lots were used, the identification of bacteria from seeds or plants was performed according to GRONDEAU *et al.* (1992).

Location of PSP at the seed level

15 samples of 40 to 50 pea seeds from a naturally infected seed lot (cultivar Belinda) were shaken for one hour in sterile water. This first wash was analyzed to detect bacteria on the seed surface. Then, seed coats were separated from cotyledons and washed apart for one hour. These washes were also analyzed to detect bacteria on cotyledon surface and on coat internal surface, mainly. Coats and cotyledons were kept 26 hours in maceration at 4°C for the release of possible internal bacteria. These washes were finally analyzed.

Location of PSP at the seed lot level

Pea seeds of 4 naturally infected lots (cultivars Belinda and Solara) were analyzed one by one. Seeds with or without lesions were distinguished. 24 to 175 seeds were individually analyzed.

Study of the relationship between seed infection, subsequent plant contamination and symptoms, using artificial seed inoculation

Pea seeds (cultivar Belinda) were vacuum-infiltrated with bacterial suspensions at different concentrations, using a marked strain. A part of the inoculated seeds was analyzed whereas the other part was sown in greenhouse. Plants were analyzed at the emergence 8 days after sowing, and diseased plants counted 15 days after sowing. Regression analysis was performed with mean numbers of bacteria per seed and, first, population levels on the plant, secondly, percentage of diseased plants.

Colonization of a plot from natural seed infection

Pea seeds from a naturally infected seed lot were treated by 2 hours soaking in a streptomycin solution (5 mg/ml), then dried. Control seeds were soaked in water. Control and disinfected seeds were sown on april the 4th, 1989, in 2 adjacent plots of 8 by 2 m each. Within each plot, 8 equal squares (1 m^2) were delimited and 4 plants in each square corner picked up for population determination 42 and 88 days after sowing.

RESULTS

Location of PSP at the seed level

For most of the samples, PSP was mainly isolated from coats. In 2 samples out of 15, cotyledons were infected by more than 80 % of the total numbers of bacteria detected. The first sample was not highly infected (mean of 20 bacteria per seed). In the second one, more heavily infected (3.5×10^4 bacteria per seed), bacteria were detected on cotyledons after one hour shaking, showing rather a surface contamination of cotyledons. So, the contamination of this seed lot appeared mainly associated with coats, on their external and internal surfaces.

Location at the seed lot level

PSP was more frequently found on seeds harboring lesions (6 to 17 % of seeds against 0 to 0.6 % for visually healthy seeds). Germination rates for such seeds from one of the seed lot used, was 57 % in average, showing that they were able to give rise to a plant and so might act as primary inoculum source. Population levels could considerably differ from one seed to another : they could range from 10 to 10^6 viable cells, attesting that contamination was not uniform over the seeds.

Study of the relationship between seed infection, subsequent plant contamination and symptoms, using artificial seed inoculation

High correlations were found between the amount of PSP inoculated per seed and, first, population levels detected on plants at the emergence, 8 days after sowing ($r = 0.94$) and secondly, on symptoms that have developed 15 days after sowing ($r = 0.96$). The quantitative seed infection seemed to be an important factor of disease severity.

Colonization of a plot from natural seed infection

42 days after sowing, 16 % of plants carried PSP in the control plot whereas only 5 % in the disinfected one. No symptoms occurred in the 2 cases. Contamination cartographies showed the origin of primary inoculum foci from seeds. Foci were randomly distributed in the plots. They were less frequent and less highly contaminated when seeds were disinfected. However, the effect of the disinfection was not total, and the 3 contaminated plants that were left sufficient to generate the epidemic : 88 days after sowing, the disinfected plot was as highly contaminated as the control one.

DISCUSSION - CONCLUSION

Contamination by PSP is not uniformly distributed over pea seeds and so, is unpredictable.

In the seed lot tested, coats were found to shelter most of the contamination. These results obtained by isolation agree with those of SKORIC (1927) by histology.

In the 4 seed lots tested, the pathogen appears to be strongly related with seeds showing lesions. Such seeds are able to give rise to plants and so, may provide primary inoculum in field. They probably escape notice because of seed treatments covering seed. Selecting visually healthy seeds would decrease the amount of primary inoculum, but we don't know to what extent.

By artificial seed contamination, we found that the amount of bacteria on plants, and later the amount of symptoms, was related with the amount of viable cells carried by the seed. WELLER and SAETTLER (1980) found that a minimum of 10^3-10^4 cells per seed of *Xanthomonas campestris* pv. *phaseoli* was required for plant infection. We could estimate that 23 % of the plants rising from the seeds carrying 5 x 10^3 bacteria would already show symptoms, and that 1 to 2 % of plants rising from seeds carrying only one bacterium would be diseased. We already knew that the percentage of infected seeds in a lot could influence disease levels in the culture (ROBERTS, 1991). Our results illustrate the fact that even with a low amount of bacteria on seed, a risk of infected plant apparition and so, of primary inoculum source, does still exist.

TAYLOR and DYE (1976) obtained a 90 % reduction of primary infection with streptomycin seed treatment (slurry) measured by symptoms recording. We have shown that seeds disinfected with streptomycin generate less plants carrying epiphytic PSP than control, and that some contaminated plants were able to serve as inoculum source for the invasion of nearly all the whole plots.

All these facts support the importance of seed infection in pea blight epidemiology : at early stages, bacterial occurrence in field is strongly governed by seed contamination, and either in natural or artificial conditions, bacteria on leaves originate from bacteria on seed

RESUME

La graisse du pois, causée par *Pseudomonas syringae* pv. *pisi* (PSP), est transmise par la semence.

La distribution des bactéries au niveau de la graine et au niveau du lot a été étudiée. Par analyse séparée des cotylédons et des téguments, nous avons trouvé que, pour le lot de graines testé, PSP était principalement associé aux téguments, soit sur la graine, soit face interne des téguments (entre téguments et cotylédons). Une analyse graine par graine sur 4 lots différents, a montré que PSP était plus fréquemment isolé des semences montrant des lésions, et qu'une semence pouvait abriter jusqu'à 10^6 cellules viables.

Pour étudier l'effet de la quantité de bactéries portée par la graine sur les populations épiphytes ultérieures et sur l'incidence de la maladie, des graines ont été inoculées, par infiltration sous vide, avec différentes concentrations d'une souche marquée. Une forte corrélation a été trouvée entre la quantité de bactéries sur les graines et les niveaux de population détectés 8 jours après le semis. La corrélation était aussi élevée entre la quantité de bactéries par graine et les symptômes qui sont apparus 15 jours après le semis.

L'apparition de PSP sur les plantes issues de graines d'un lot naturellement contaminé, a été étudiée en comparaison avec une désinfection des graines à la streptomycine. La distribution au hasard du pathogène dans les semences a fourni des foyers de contamination primaire. Moins de foyers ont été observés 42 jours après le semis dans la surface désinfectée, mais, 88 jours après le semis, la dissémination rapide de PSP a envahi une forte proportion des plantes dans tout l'essai. Aucun symptôme n'a été observé pendant toute la durée de l'expérimentation.

Les expérimentations conduites appuient le fait que le rôle de l'infection de la semence est crucial dans l'épidémiologie de la graisse du pois.

MOTS CLE : *Pseudomonas syringae* pv. *pisi*, pois, contamination de la semence, description de la contamination

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Latent infections of pectolytic *Erwinia* spp. on seed potato tubers

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ABSTRACT

In 1988, 89 and 90 a total of 276 seed potato tuber lots, produced in Belgium or in neighbouring countries, were analysed by incubation at 22 °C and 100% RH with the help of the 'Erwinia kit' to reveal latent contaminations by soft rot bacteria. After 14 day incubation, 19 lots showed no rotting, 124 lots presented up to 20 % of tubers affected by soft rot and the remaining 133 lots showed rotting ranging between 21 and 100%. The same distribution of infection frequency was observed for the three years. Cultivars Kennebec, Lola, Charlotte and Désirée showed the highest contamination rates. Up to 20% of the tubers of some minituber lots already showed infection by soft rot bacteria. Percentage of latent infections rapidly increased after one or two multiplication cycles in the fields. The grading process induced a 5 to 8 fold increase in the percentage of rotting after 14 days incubation. In 1991, a good correlation was observed between the percentage of soft rot after 6 days incubation (SR6), noted for the seed tubers, and the occurrence of blackleg (BL) in the field (%BL = 0.46 SR6 + 0,63). Out of 246 *Erwinia* spp. strains isolated from decayed seed tubers on improved cristal violet pectate medium, 152 were identified as *E. carotovora* subsp. *atroseptica* (Eca), 78 as *E. carotovora* subsp. *carotovora*, 7 as *E. chrysanthemi* and 9 as undefined *Erwinia* spp. Only 7 strains of Eca out off 70 tested, reacted positively with SANOFI 36681 antiserum raised to the serogroup I.

Keywords : Erwinia carotovora subsp. carotovora, E. c. subsp. atroseptica, E. chrysanthemi, blackleg, soft rot.

INTRODUCTION

Latent infections of potato tubers by pectolytic Erwinia spp. (*Erwinia carotovora subsp. atroseptica* (Eca), *Erwinia carotovora subsp. carotovora* (Ecc) and *Erwinia chrysanthemi* (Ech)), can evolve into blackleg and soft rot, diseases responsible for downgrading and refusal in seed tuber certification. In most countries, certification tolerances are still based on the expression of blackleg in the crop.

However, blackleg incidence in daughter crop can not be accurately predicted from the amount observed in the mother crop. Moreover, blackleg tolerance may not be a warranty that the amount of soft rot during storage or transport will not exceed the tolerance because the disease expression is unpredictable due to environmental effects. Although other sources of inoculum exist, the mother tuber is potentially the principal source of contamination for the growing crop (PEROMBELON & KELMAN, 1980). In 1987, because of the growing interest of farmers in producing high quality potato seed tubers and the poor knowledge concerning control possibilities of the disease, our laboratory developed a simple incubation method, the "Erwinia kit", to reveal the latent infections. The paper presents the results obtained by analysing seed potato lots produced in Belgium and in neighbouring countries.

MATERIALS AND METHODS

Detection of latent infections. Latent infections by soft rot bacteria were revealed with the help of the "Erwinia kit" as described by DELFOSSE *et al.*, 1992. Tubers were observed after 6, 10, 14 days incubation to assess the percentage of rotting.

Isolation of the soft rot bacteria. Decayed tubers, taken after incubation, are washed under tap water to eliminate the rotted tissues. A small cube (1 or 2 mm side) is taken from the border line of intact tissues and then squeezed into a tube containing 2 ml sterile deionized water. Thirty µl of a 100 fold dilution of the initial isolation suspension is poured on improved crystal violet pectate medium (CVP) in Petri dishes (WOODWARD & ROBINSON, 1990). The suspension is spread on the surface of the medium by shaking horizontally the dishes containing 3 to 5 small sterile glass balls (3mm diameter). Colonies rapidly forming deep pits on CVP after incubation at 27 °C are suspected of being *Erwinia* spp. and are isolated on nutrient agar for further characterization.

Bacterial characterization. Strains detected during isolation are retested on CVP for pectolytic activity. Only strains reproducing pits after 48 h are further tested for *Erwinia* spp. criteria (BRADBURY, 1970). Differentiation in species and subspecies is made on the basis of acid production from dulcitol, lactose, palatinose and α methyl-D-glucoside, production of indol from tryptophane and reducing substances from sucrose (RUDOLPH *et al.*, 1990, SANDS & DICKEY, 1978). Strains identified as ECA are tested by indirect immunofluorescence (IIF) with "Sanofi" antiserum raised to the serogroup I (strain NCPPB 1526), (DE BOER et al. , 1979).

RESULTS AND DISCUSSION

In 1988, 1989 and 1990, a total of 276 seed lots were analysed with the help of the 'Erwinia kit'. After 14 day incubation, 19 lots showed no rotting, 124 lots presented up to 20 % of tubers affected by soft rot and the remaining 133 lots showed rotting ranging between 21 and 100%. Despite variations in climatic conditions, almost the same distribution in categories of infection was observed for each year (Fig.1). Twenty-four different cultivars were analysed, the most frequent are listed in Table 1. Cv. Kennebec showed the highest percentage (up to 100%) of tubers affected by soft rot after 14 days incubation. Cvs Charlotte, Désirée and Lola can also be affected by high contamination rates. However, some lots appeared very little contaminated, regardless of the cultivar and of the certification class. Commercial minituber lots, grown in greenhouses, were shown to present up to 21.5 % of tubers affected by soft rot after 14 days incubation. The percentage of latent infections, revealed by soft rot, rapidly increased after 1 or 2 multiplication cycles in the field (TABLE 2). The 'Erwinia kit' was a useful tool to assess the effect of handling and grading on latent infections. Handling and grading can be responsible, respectively, for a 5 and 8 fold increase in percentage of tubers affected by soft rot after incubation (Fig. 2). NAUMANN *et al.*, cited by HOFFMAN *et al.* (1985), also observed a 2 to 44 fold increase in percentage of rotting induced by the handling and the grading processes. The expression of latent contaminations are possibly triggered by shocks during these processes. Actually, a lot of apparently healthy tubers (cv. Charlotte), tumbled for 10 minutes in a RETSCH mixer, showed no apparent injury after this treatment, but appeared up to 30 fold more affected by soft rot after incubation (unpublished data). In 1991, the percentage of blackleg (BL) in the fields was correlated with the percentage of tubers affected by soft rot observed after 6 day incubation (SR6) for 14 seed lots (% BL=0.46 SR6+0.63), (Fig. 3). The spring of 1991 was humid and cold and numerous thunder showers occurred later on. These climatic conditions are particularly favourable for the development of blackleg.

Out of 246 *Erwinia* spp., isolated from decayed seed tubers after incubation with the help of the 'Erwinia kit', 152 were identified as ECA, 78 as ECC, 7 as Ech and 9 as undefined *Erwinia* spp. Only 7 strains of Eca out of 70 tested, reacted positively in IIF with the antiserum raised to the serogroup I of *E. carotovora* sp.

In conclusion, the "Erwinia kit" appears to be a useful and simple tool to reveal latent infections by the three *Erwinia spp.*, to study the epidemiology of the disease, to help seed growers to choose lots for further multiplications and to assess possibilities of control (Delfosse et al., 1992).

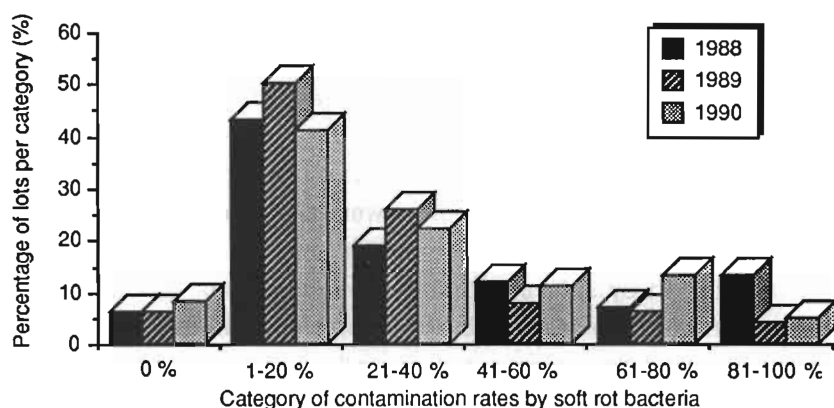

FIGURE 1 : Percentage of commercial seed lots per category of contamination rates by soft rot bacteria. Latent contaminations are revealed by soft rot after 14 days of incubation with the help of the 'Erwinia kit' (Number of lots analysed : 1988 : 41 lots, 1989 : 115 lots, 1990 : 120 lots).

TABLE 1 : Latent infections revealed by soft rot after incubation according to the cultivar for pre-basic, basic and certified seeds.

Year	Cultivar	Number of lots analysed	Percentage[1] of tubers affected by soft rot minimum	mean	maximum
1989	Bintje	13	1	15	36
	Charlotte	18	0	14	40
	Désirée	27	0	21	73
	Kennebec	20	4	47	100
	Ostara	6	0	15	36
	Sirtema	6	1	4	11
	Others[2]	25	0	24	78
	Total	115	0	20	100
1990	Bintje	8	1.5	10	21
	Charlotte	9	1.5	27	75.5
	Désirée	14	1.5	17.1	45
	Kennebec	41	0	42	94
	Lola	7	8.5	43.7	68.5
	Others[2]	28	0	12.2	91.5
	Total	107	0	25	94

1 Percentage of tubers affected by soft rot after 14 days of incubation with the help of the 'Erwinia kit'. For each lot, 70 apparently healthy tubers were incubated.

2 For these 17 other cultivars less than 6 lots were analysed.

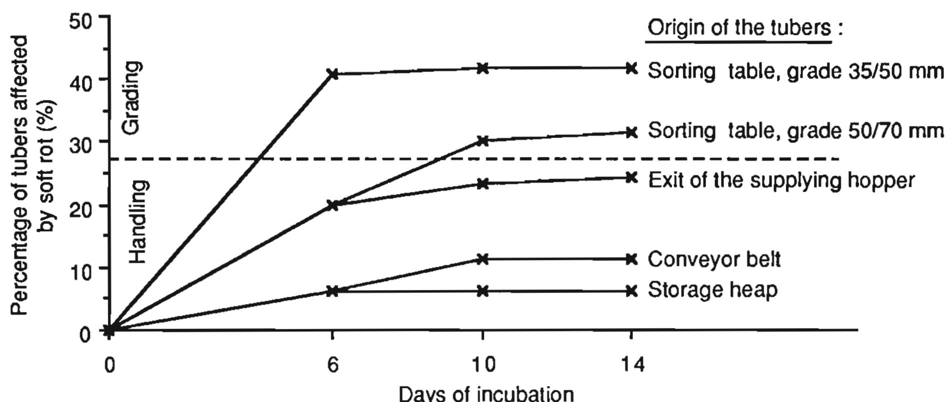

FIGURE 2 : Effect of handling and grading on the occurrence of soft rot revealing latent infections after 14 days of incubation. For each handling or grading step, 70 apparently healthy tubers were incubated with the help of the 'Erwinia kit'. Cultivar Kennebec, seed class SE.

TABLE 2 : Latent infections revealed by soft rot after incubation of minituber lots and of seed lots, coming from minitubers, after 1 or 2 multiplication cycles in the fields.

	Minitubers			Crop multiplication 1 year			Crop multiplication 2 years		
year	Cultivar	Ref.[2]	S.R.[1]	Cultivar	Ref.[2]	S.R.[1]	Cultivar	Ref.[2]	S.R.[1]
1990	Eba	684	0	Bintje	724	14.5	Désirée	720	3
	Jaerla	707	3	Désirée	723	17	Désirée	721	18.5
	Jaerla	708	3	Kennebec	725	37	Kennebec	718	70
	Jaerla	709	0	Ker pondy	726	41.5	Kennebec	719	69
	Kennebec	685	14						
	Kennebec	632	3						
	Kennebec	703	0						
	Kennebec	704	1.5						
	Majestic	683	4.5						
	Primura	705	0						
	Red Pontiac	701	16						
	Red Pontiac	702	21.5						
	Sirtema	706	0						
1991	Claustar	926	13				Kennebec	979	28
	Claustar	927	9						
	Claustar	928	7						
	Jaerla	920	0						
	Jaerla	921	1						
	Jaerla	922	0						
	Primura	923	20						
	Primura	924	3						
	Primura	925	9						
	Red Pontiac	917	7						
	Red Pontiac	918	6						
	Red Pontiac	919	3						
	Mean		5.8			27.5			37.7

1 Percentage of tubers affected by soft rot after 14 days of incubation with the help of the 'Erwinia kit'. For each lot, 70 apparently healthy tubers were incubated.

2 Lot reference.

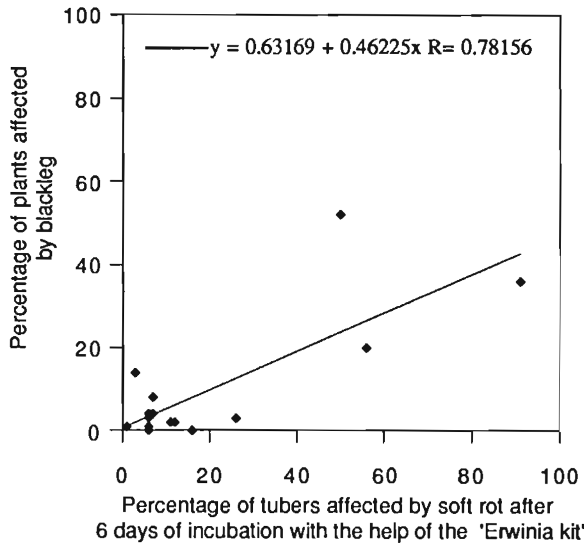

FIGURE 3 : Relation between latent infections revealed by the "Erwinia kit" and the occurrence of blackleg in the field (14 lots observed in July and Augustus 1991)

ACKNOWLEDGMENTS

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Populations of *Pseudomonas viridiflava, P. cepacia*, pectinolytic bacteria, and total bacteria from soils, irrigation water, onion foliage and onion bulbs in Georgia (USA)

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Agar media, (T-5 for isolation of Pseudomonas viridiflava, total pseudomonads, and total bacteria, CVP for isolation of pectinolytic bacteria, and TBT for isolation of P. cepacia) were used to monitor bacterial populations in soil, water and plant parts in onion-growing areas from October, 1991 to May, 1992. P. viridiflava was detected as an epiphyte in association with frost on onion leaves in January, 1992, which was 3-4 weeks prior to the observation of symptoms. Pathogenic bacteria were not detected in soils, irrigation water, or from volunteer soybean plants sampled from seedbeds or onion fields in the fall of 1991. There was no significant difference between treatments (various levels of methyl bromide, chloropicrin, Telone C17, or no treatment) immediately after fumigation or during any sampling period from October to May as regards to the number of cfu/g of soil of total bacteria, or fluorescent pseudomonads, assayed on T-5 agar medium or in the number of cfu/g of soil of pectinolytic bacteria monitored on CVP agar medium. Fumigation treatments had no significant control of bacterial streak and rot caused by P. viridiflava.

INTRODUCTION

Bacterial streak and rot of onion (Allium cepa L.), caused by Pseudomonas viridiflava (Burkholder) Dowson, has been a problem in Georgia since 1990 (GITAITIS et al., 1991a). Water-soaked streaks develop on upper portions of leaves and the bacterium rots the bulb and the base of leaves. The disease has been particularly severe under cool, wet conditions that have occurred in the winter and early spring. Little is known regarding the epidemiology of this disease, or the bacterium's interaction with cultural practices, the environment, or microorganisms associated with postharvest decay (COTHER & DOWLING, 1986; GITAITIS et al., 1991b). A semi-selective medium was needed to suppress nontarget microflora yet provide for suitable growth and differentiation of colonies of P. viridiflava.

MATERIALS AND METHODS

A semi-selective medium (T-5) was developed based on media previously published (SANDS & ROVIRA, 1970; SIMON et al., 1973). Each liter of T-5 contained 5.0 g of sodium chloride, 1.0 g of monobasic ammonium phosphate, 1.0 g of dibasic potassium phosphate, 0.2 g of magnesium sulfate septahydrate, 3.0 g of D-tartaric acid, 0.01 g of phenol red, 20 g of agar, and deionized water. The pH was adjusted to pH 7.2 with 1 N sodium hydroxide. The medium was sterilized at 121 C for 15 min. After media cooled, 10 mg of bacitracin, 6 mg of vancomycin, 75 mg of cycloheximide, 45 mg of novobiocin, and 50 mg of penicillin G were added aseptically.

Strains of P. viridiflava were isolated from onions in Georgia; obtained from J. B. Jones, University of Florida (original hosts were tomato and parsnip); or obtained from the ATCC (original host was bean (BURKHOLDER, 1930)). Strains were cultured on King's medium B (KMB) (KING et al., 1954) prior to all tests. Plating efficiences were determined by comparing colonies on T-5 with growth and development on KMB. Recovery from soil was evaluated by adding 1 ml of a suspension (1 x 10^5 cfu/ml) of P. viridiflava to 10 g of nonsterile soil suspended in 100 ml of PBS. Efficiency of recovery was evaluated on plates of KMB and T-5. The test was conducted in soils that had either no history or a 16-yr history of onion production. To further reduce competition by nontarget microorganisms, effect of incubation temperature was evaluated. Known populations of P. viridiflava

were cultured on KMB and T-5 at 30, 25, 15, 10, and 5 C. Plating efficiencies were determined. All tests were replicated three times.

The influence of soil fumigation on root and bulb rot caused by fungi, P. viridiflava, and other bacteria was studied at two sites in the Vidalia onion-growing region. Treatments consisted of 67% methyl bromide-33% chloropicrin at 88 and 127 kg/ha, chloropicrin at 85 and 124 kg/ha, 98% methyl bromide-2% chloropicrin at 162 and 258 kg/ha, 77.9% 1,3-dichloropropene-16.5% chloropicrin (Telone C-17) at 56 L /ha, and a nontreated control. Experimental design was a randomized complete block with three replications. Composite soil samples, onion foliage, onion bulbs, irrigation water, and volunteer soybean plants were sampled periodically from October, 1991 through April, 1992, plated on to T-5, CVP (CUPPELS & KELMAN, 1974), and TBT (HAGEDORN et al., 1987), and incubated either at 26 or 5 C.

The effects of fertility on disease were studied in the field in Tifton. Fertility treatments consisted of standard (total of 850 kg) and excessive (1980 kg) rates of fertilizer applied over a 20 wk period. Plants were inoculated with P. viridiflava on 18 December, 1991 and on 13 January, 1992. Foliar disease ratings were made in the field from January through April, and bulb ratings were made at harvest.

RESULTS

When incubated at 26 C, plating efficiencies on T-5 for four strains of P. viridiflava ranged from 67 to 120% (x = 87.8%). In the soil recovery assay, P. viridiflava was recovered (x of actual value = 2.6×10^3 cfu/ml, whereas the expected value was 1×10^3 cfu/ml) on T-5 from soil that had no history of onion production. High populations of saprophytic bacteria and fungi prevented recovery of the target organism from KMB. Soil from areas with a long history of onion production produced considerable numbers of nontarget bacteria and fungi on T-5 plates which made recovery of P. viridiflava more difficult. When compared to incubation at 30 C, mean plating efficiencies on T-5 for three strains of P. viridiflava were 86, 82, 86, and 82% at 25, 15, 10, and 5 C, respectively and on KMB were 98, 100, 99, and 100% at 25, 15, 10, and 5 C, respectively. When recovery of bacteria from eight soil and three irrigation water samples was evaluated, there

was an approximate 1000 fold reduction of nontarget microorganisms on T-5 incubated at 5 C compared to 30 C.

From October, 1991 to April, 1992, P. viridiflava was not detected in any soil, soybean, or irrigation water sample. The only time the bacterium was detected before symptoms were evident was as an epiphyte in association with frost on onion leaves in January, 1992. Erwinia chrysanthemi and P. cepacia were recovered occasionally from soil, irrigation water, and onion bulbs. All three pathogens and many saprophytic bacteria were recovered as endophytes in bulb interiors.

Regardless of location, there was an increase in populations of pectinolytic bacteria recovered on CVP plates over time. Mean pectinolytic, soilborne, bacterial populations were 1.4×10^3, 6.3×10^3, 3.9×10^4, and 5.0×10^4 cfu/g of soil in October, November, January and March, respectively. In a loamy clay soil (site A), populations of total bacteria at 26 and 5 C, and fluorescent pseudomonads at 5 C declined from October through March, whereas fluorescent pseudomonads growing at 26 C increased from January to March. In a loamy sand soil (site B), populations of total bacteria and fluorescent pseudomonads declined from October to January at both 26 and 5 C, then all increased significantly from January to March. Fumigation treatments did not affect foliar or bulb disease severity. There were significantly higher numbers of endophytic, nonpathogenic bacteria in bulbs grown in soils treated with the higher rate of chloropicrin. Soils treated with higher rates of chloropicrin, either alone or in combination with methyl bromide, produced higher yields of onions.

Excessive amounts of fertilizer increased bacterial streak and rot severity compared to what was observed in onions receiving the standard, recommended levels of fertilizer. Although disease levels in onions treated with excessive levels of fertilizer were higher in all months, the logistic transformation of the disease progress curves for both treatments indicated a very similar rate (r) of disease development, r= 0.049 for excessive, and r= 0.057 for standard fertilizer rates. Early in the epidemic (February), P. viridiflava was recovered 100% of the time from both typical symptoms and from necrotic tips (dieback) of leaves in plants receiving excessive amounts of fertilizer. Plants treated with standard levels of

fertilizer had no typical symptoms but did exhibit the tip dieback. Attempts to recover P. viridiflava from 20 such samples failed to produce the bacterium. In March, P. viridiflava was again isolated in 100% of the samples displaying both symptom types in plants receiving excessive fertilizer, in 100% of the samples displaying typical symptoms in plants receiving standard fertilizer levels, and in 60% of samples with tip dieback in plants receiving the standard fertilizer treatment.

DISCUSSION

The semi-selective medium T-5 can be useful in the recovery of P. viridiflava from various sources. When first evaluated, the medium was nearly 100% selective when known populations of P. viridiflava were added to soil. Unfortunately, the soils used in the first tests had a different cropping history than those for which the medium was intended. When used in epidemiologic studies in onion-growing areas, the medium was not as efficient as we desired in eliminating nontarget microorganisms. An observation of refrigerated onions that continued to rot led us to the use of medium T-5 in conjunction with incubation at 5 C for enhanced isolation of P. viridiflava.

In spite of our best efforts, we failed to detect P. viridiflava in soil, irrigation water or on volunteer soybean plants. It is possible that the bacterium survives in association with weeds or other plants outside of the area and then is disseminated in to onion fields, the bacterium may be seedborne, or finally, the source of inoculum may be at levels below which we could detect.

Several other pathogenic bacteria, including P. cepacia and E. chrysanthemi were recovered but did not cause significant disease problems in the field. Fumigation with any of the chemicals tested had little effect on bacterial streak and rot, other bacterial diseases, or on total bacterial populations in the soil. This could be due to either the lack of bactericidal activity by the products, an ineffective method of application (water was used to seal the surface as no plastic cover was used), or because the microorganisms reentered (possibly in irrigation water) and rapidly recolonized the soil.

The best hope of managing this disease may be the use of good cultural practices. In particular, the use of standard, recommended levels of fertilizer offer some disease control. Often, onion growers apply excessive levels of fertilizer during winter months to promote rapid growth for early harvest and the highest

prices. Unfortunately, this practice also predisposes the plant to disease problems. In 1992, disease severity and incidence has not been as great as in the recent past. However, losses may have been more subtle. Rather than colonizing and eventually rotting the interior of the bulb, in many instances the bacterium simply destroyed outer leaves, which resulted in smaller onions in 1992.

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Epidemiological aspects of lettuce bacterial spot induced by *Xanthomonas campestris* pv. *vitians* [1]

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INTRODUCTION

In the years 1988 and 1989 *Xanthomonas campestris* pv. *vitians* (Brown) Dye (XCV) caused severe damage to cool season lettuce crops in southern Italy (PENNISI & PANE, 1984). In both years the epidemics occurred in November after prolonged rainfall or unusually early frosts. Iceberg is the most widely cultivated lettuce type in this period of the year and the crops that were affected at the stage of head formation were completely destroyed. Until then disease had occurred only episodically, in limited areas and never with such violence (RAGOZZINO, 1969).

The literature concerning lettuce bacterial leaf spot is not copious and several aspects of the disease cycle are still obscure or objects of hypotheses (OHATA *et al.*, 1982; PATTERSON, 1986). Investigations were conducted on some epidemiological aspects of the disease in order to obtain suggestions for its prevention and control. A virulent spontaneous rifampicin-resistant mutant of XCV and the indirect IF technique were the main tools that allowed some of the results of this study to be obtained.

MATERIALS AND METHODS

Survival of XCV in the soil. Two samples of cultivated soil, natural and autoclaved, were saturated with a suspension (1 x 10^{10} cfu ml^{-1}) of the Rif^r mutant and stored in Petri dishes, in the dark at room temperature. At different intervals the surviving bacteria were counted by soil dilution on NAS containing cycloheximide 250 ppm and rifampicin 100 ppm (NAScr). The presence of the pathogen was monitored by indirect IF (EREN & PRAMER, 1966) in soil samples collected monthly in a field where inoculated lettuce was cultivated during Spring 1990. The debris of the culture was buried in May.

Seed transmission of the disease. Seeds of cultivar Nabucco, contaminated with a suspension of Rif^r mutant (1x10^8 cfu ml^{-1}) were sown, in February 1990, in a sterile garden soil and kept in the greenhouse. Six weeks later

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the plantlets were transferred to a field far away from other lettuce crops and where this vegetable had never been grown. In August the seed produced by the plants were harvested and separated from the chaff by a pneumatic device. From the moment of emergence the plants were periodically examined for XCV presence by IF and by plating the leaf washings on NAScr. Similar procedures were followed to detect the bacterium on the seed.

Detection of XCV in commercial seeds. Twenty-two seed lots of Iceberg type cultivars were treated according to Schaad's extraction protocol (SCHAAD, 1989). Final pellets, properly diluted were plated on NASc and examined by IF. The rabbit antiserum employed in this research positively reacted with all of the twenty XCV isolates tested. Among the twenty-six plant pathogenic and non pathogenic bacteria assayed, the antiserum cross rected only with strains of *Xanthomonas campestris* pv. *campestris*; its specificity to XCV was satisfactorily improved by an absorption treatment with *Xanthomonas campestris* pv. *campestris*.

Seed treatments. Artificially contaminated seeds were treated with chemicals applied as slurries, with NaClO, hot water and dry heat. Chemicals, concentrations, temperatures and duration of treatments are reported in Tab. 1. One gram of seed was used for each condition. After each treatment 200 seeds were distributed into four plates containing NAScr and observed for bacterial growth while 50 seeds were put on water agar to check germination.

Influence of plant age and fertilizers on incidence and intensity of the disease. A field trial was carried out in the period September-December 1991 where plants of three different ages (4, 6 and 8 weeks), with or without the addition of ordinary amounts of nitrogen (150 U/ha) and phosphorus (100 U/ha), were sprayed with XCV. The test was carried out according to a split plot design replicated four times; fertilizers were assigned to the main plots and the age of the plants at the moment of bacterial inoculation was the subplot factor. Plants were monitored after 2, 3, 5, 7 and 9 weeks and were assigned to four classes of symptom severity (1=no symptoms; 2=light symptoms on external leaves; 3=serious symptoms on external leaves; 4=seriuos symptoms on internal leaves). The percentage data of diseased plants for each class of symptom severity were transformed into angular values before the analysis of variance was applied. The means of treatment were compared with the Duncan multiple range test. Mean values followed by the same letters are not different at $P = 0.01$.

RESULTS

Survival in the soil. As shown in Graph. 1 the number of survivors in the sterile soil gradually decreased from 1×10^9 cfu g^{-1} dry soil to a few dozen in about ten months; in the natural soil the number of the bacteria rapidly dropped to similar levels in three months and after ten months bacteria became undetectable. In the soil of the cultivated field, bacteria were detected by IF from March to October but not later in November and December.

Seed transmission. Lettuce plants, originating from artificially contaminated seed were analysed by IF and were shown to host XCV epiphytically for a long phase. In the field many of the plants, when near maturity, showed symptoms following rainfall. Bacterial populations were particularly abundant on the inflorescence. The Rif^r mutant was isolated from symptomless leaves, from lesions and, later, from about 11% of the harvested seed.

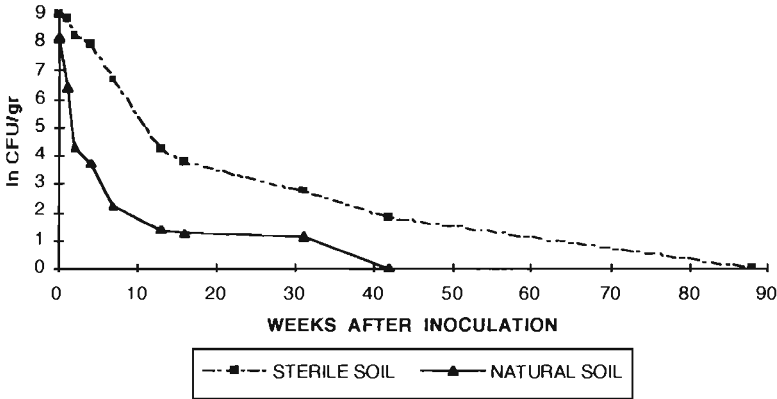
Detection of XCV in commercial seed. Of the twenty-two seed lots processed, eight showed contamination by the bacterium when examined by IF. Three lots were heavily contaminated, from one of which XCV was successfully

recovered. In all other cases attempts to obtain colonies of the bacterium failed because of overgrowth of saprophytic seed microflora and/or of scarce viability of XCV cells.

Seed treatment. The effect of the different treatments applied to the seeds are reported, in order of increasing efficacy, in Tab.1. All the chemicals were applied as slurries because of the lower toxicity to the seeds when given in this form rather than as suspensions or solutions. Only firestop 10000 ppm and NaClO (1% Cl^-) for 60 min completely eradicated bacteria from seeds; both treatments left seed viability unaffected. All other treatments failed to give satisfactory disinfection; in particular heat treatments markedly reduced seed germination.

Influence of plant age and fertilizing on incidence and intensity of the disease. Tab.2 shows the percentage distribution in the four classes of symptoms of the diseased plants as recorded in the subsequent five surveys. Larger numbers of older plants are affected than younger ones and plants are increasingly affected with ageing. Severity of simptoms too seems positively correlated to the age of the plants; with ageing a shift of the percentage of the diseased plants can be observed towards the higher class of symptoms.Higher percentages of diseased plants and more severe symptoms may also be observed among those plants that received both fertilizers while less fed plants seem to be least affected.

Graph 1. SURVIVAL OF XCV IN THE SOIL (laboratory conditions)



DISCUSSION

Our data suggest that lettuce bacterial spot should be considered a seed-borne disease: from artificially contaminated seeds plantlets were obtained that were epiphytically colonized by XCV; many of these plants became diseased when transferred to the field, and produced contaminated seed. In 1989 and 1990 twenty-two samples of Iceberg type lettuce seed were examined by IF and eight were found to host cells of the pathogen. These findings, together with the evidenced ability of XCV to live epiphytically on the leaves can account for the long distance spread of the disease and the rapid development of serious epidemics when favourable environmental conditions occur.

Tab. 1: SEED TREATMENTS

TREATMENT		CONCENTRATION	% CONTAMINATED SEEDS
bronopol		1000 ppm	100.0 a
bronopol		2000 ppm	100.0 a
streptomycin		200 ppm	100.0 a
streptomycin		500 ppm	100.0 a
streptomycin		1000 ppm	100.0 a
kasugamycin		100 ppm	100.0 a
kasugamycin		500 ppm	100.0 a
kasugamycin		1000 ppm	100.0 a
kasugamycin		2000 ppm	100.0 a
hot water	42°C 60 min.		100.0 a
dry heat	70°C 24 hours		100.0 a
dry heat	70 °C 48 hours		100.0 a
dry heat	70° C 72 hours		98.7 a
dry heat	70° C 96 hours		98.7 a
mancozeb		3000 ppm	97.5 a
mancozeb		6000 ppm	97.5 a
streptomycin		2000 ppm	97.5 a
Cu hydroxide (Cu^{++} 30%)		7000 ppm	97.5 a
hot water	44°C 60 min.		97.5 a
cobox		6500 ppm	96.2 a
Cu hydroxide (Cu^{++} 30%)		14000 ppm	88.7 ab
cobox		13000 ppm	77.5 b
hot water	46° C 60 min.		60.0 c
bronopol		5000 ppm	51.2 cd
firestop		2000 ppm	43.7 de
cobox		32000 ppm	37.5 e
thiram		16000 ppm	37.5 e
Cu hydroxide (Cu^{++} 30%)		35000 ppm	25.0 f
thiram		24000 ppm	17.5 fg
NaClO 1%	20 min.		15.0 fg
thiram		32000 ppm	10.0 gh
firestop		4000 ppm	8.7 gh
NaClO 1%	40 min.		1.2 h
firestop		10000 ppm	0.0 h
NaClO 1%	60 min.		0.0 h

Data followed by the same letters are not significantly different according to Duncan test (P=0.01)

Tab. 2: Percent distribution of diseased plants in the four classes of symptoms as influenced by age and fertilizing

Elapsed time from inoculation	Treatment		Symptomatological class 1	2	3	4
2 weeks	Plant age at inoculation (weeks)	8	97.8	2.2	0.0	0.0
		6	100.0	0.0	0.0	0.0
		4	100.0	0.0	0.0	0.0
	Fertilizers	$N_0 P_0$	99.2	0.8	0.0	0.0
		$N_0 P_1$	100.0	0.0	0.0	0.0
		$N_1 P_0$	99.6	0.4	0.0	0.0
		$N_1 P_1$	98.3	1.7	0.0	0.0
3 weeks	Plant age at inoculation (weeks)	8	90.0a	10.0b	0.0	0.0
		6	97.2b	2.8a	0.0	0.0
		4	99.7b	0.3a	0.0	0.0
	Fertilizers	$N_0 P_0$	95.4	4.6	0.0	0.0
		$N_0 P_1$	96.2	3.8	0.0	0.0
		$N_1 P_0$	97.5	2.5	0.0	0.0
		$N_1 P_1$	93.3	6.7	0.0	0.0
5 weeks	Plant age at inoculation (weeks)	8	30.0a	56.6b	11.2b	2.2a
		6	50.3b	49.7b	0.0a	0.0a
		4	70.4c	29.3a	0.3a	0.0a
	Fertilizers	$N_0 P_0$	66.2c	33.8a	0.0a	0.0a
		$N_0 P_1$	53.3b	45.8b	0.9a	0.0a
		$N_1 P_0$	50.4b	44.2b	5.4b	0.0a
		$N_1 P_1$	31.7a	57.1c	7.9b	3.3a
7 weeks	Plant age at inoculation (weeks)	8	0.0	65.6a	17.5b	16.9b
		6	0.0	94.1b	5.6a	0.3a
		4	0.0	97.2b	2.8a	0.0a
	Fertilizers	$N_0 P_0$	0.0	92.9b	7.1a	0.0a
		$N_0 P_1$	0.0	89.6b	7.9a	2.5a
		$N_1 P_0$	0.0	87.9b	6.7a	5.4a
		$N_1 P_1$	0.0	72.1a	12.9b	15.0b
9 weeks	Plant age at inoculation (weeks)	8	0.0	11.6a	25.0a	63.4b
		6	0.0	35.9b	62.5b	1.6a
		4	0.0	80.9c	19.1a	0.0a
	Fertilizers	$N_0 P_0$	0.0	44.6ab	43.3b	12.1a
		$N_0 P_1$	0.0	42.1ab	31.9ab	20.0ab
		$N_1 P_0$	0.0	50.0b	26.7a	23.3bc
		$N_1 P_1$	0.0	34.6a	34.2ab	31.2c

Data followed by the same letters are not significantly different according to Duncan test (P=0.01)

N_0 = no nitrogen
P_0 = no phosphorous
N_1 = nitrogen 150 u/ha
P_1 = P_2O_5 100 u/ha

1 = no symptoms
2 = light symptoms on external leaves
3 = serious symptoms on external leaves
4 = serious symptoms on internal leaves

Like many other phytopathogenic bacteria, XCV can survive in the soil for more or less prolonged periods of time; duration of survival of infectious populations probably depends on the biological activity of soil microflora i.e. its capacity to degrade crop debris. Bacteria were detected by IF five months after crop harvesting; perhaps in natural conditions, their survival can be long enough to account for some limited infections if a new lettuce crop is planted within the year.

In southern Italy environmental conditions which are very favourable to outbreaks of the disease occur between the end of October and November when temperatures are still moderate and rainy periods are frequent. In these months lettuce plants of autumn-winter crops progress from their juvenile stages, that are somewhat resistant, to maturity that is, on the contrary, a very susceptible stage. If large epiphytic populations of XCV had the chance to build up in the crop in September-October, ruinous epidemics could only be controlled with great difficulty.

It is clearly essential to start with clean, healthy seed as is demonstrated by the results of our field tests and analyses carried out on commercial seeds.

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Assessment of yield losses in almond due to *Xanthomonas campestris* pv. *pruni* (Smith) Dye

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ABSTRACT

The loss in green fruit yield (GFY) per tree has a strong correlation with the leaf spot intensity (r = 0.992) and fruit gummosis incidence (r = 0.989). Ninety height per cent of the total variation in GFY can be estimated by the linear function Y = 2.94 + 1.378 X1 - 2.239 X2 where X1 is leaf spot intensity and X2 gummosis incidence. Loss in 100 green fruit weight (GFW) and 100 fresh nut weight (FNW) in response to varying gummosis severity has been calculated by linear model. With each unit increase in gummosis severity, in the range of 1-5, the loss in 100 GFW is expected to fall between 18.04 $\pm$ 4.28, 18.11 $\pm$ 5.34 and 17.48 $\pm$ 3.17 per cent in cv. Afghanistan Seedling, Merced and Non Pareil, respectively at P = 0.05. The corresponding figures for FNW are 12.48 $\pm$ 0.77, 13.81 $\pm$ 2.65 and 14.33 $\pm$ 5.99, respectively.

KEYWORDS

Xanthomonas campestris pv. *pruni*, Bacterial leaf spot, Almond, *Prunus dulcis*, Losses.

INTRODUCTION

Xanthomonas campestris pv. *pruni* (Smith) Dye, the incitant of bacterial leaf spot and fruit gummosis in almond (*Prunus dulcis* (Mill)) Webb has been recorded from New Zealand (YOUNG, 1977) and India (JINDAL *et al.*). The bacterium causes substantial losses in green fruit yield (GFY) particularly in the lower hills. No information is at present available on the extent of losses caused by the bacterium so an attempt have been made in this respect.

MATERIALS AND METHODS

The experiment was conducted on 8 year old plants of 3 cultivars of almond viz., Afghanistan Seedling, Merced and Non Pareil in the University orchard during 1986, 1987 and 1991. The data on the disease severity on leaves were

recorded on 0.5 scale based on per cent area under lesions i.e. 0 = 0.0, 1 = 0.1-1.0, 2 = 1.1-5.0, 3 = 5.1-20.0, 4 = 20.1-50.0 and 5 > 50.0. The gummosis severity on fruits was recorded following 0.5 scale described by Jindal *et al.* (1990).

Effect of leaf spot intensity (LSI) and gummosis incidence :

Thirty trees of cv. Merced with varying levels of disease were selected and categorised on the basis of LSI and GI. The data on GFY per tree and 100 fruits selected at random were recorded and per cent loss in GFY and GFW was calculated as :

$$\text{Per cent loss in yield} = \frac{\text{Yield of healthy tree - Yield of a tree with particular infection}}{\text{Yield of healthy tree}} \times 100$$

Multiple linear regression equation Y = a + b1X1 + b2X2 were fitted where Y = loss in GFY/tree or loss in 100 GFW is LSI, X2 is GI and b1 and b2 are regression coefficients.

Effect of gummosis severity (GS) :

Green fruits of cv., Afghanistan Seedling, Merced and Non Pareil showing different levels of GS were categorised into 6 categories on the basis of 0.5 scale. The weight of 100 green fruits and 100 fresh nuts (FNW) of each category were recorded and per cent losses were estimated as described above. Simple linear regression Y = a + bX taking loss in GFW and FNW as dependant variable (Y) and GS as independent variable (X) for each cultivar were fitted. Homogeneity of regression coefficients tested by general linear test method. Homogeneity of correlation coefficients were also worked out.

RESULTS AND DISCUSSION

The loss in GFY and its components in relation to different disease levels have been described by linear model.

Effect of leaf spot intensity and fruit gummosis incidence :

Simple correlation analysis of the data on loss in GFY tree has a strong correlation with the LSI (r = 0.992) and GI (r = 0.989). The corresponding figures for loss in 100 GFW are 0.984 and 0.978, respectively. Ninety height per cent of

the total variation in GFY/tree and 97.4 in GFW can be accounted for by the linear function Y = 2.94 + 1.378 X1 - 2.239 X2 and Y = 7.907 + 2.010 X1 - 0.830 X2, respectively, where X1 is LSI and X2 is GI at P = 0.05. Strong correlation of loss in GFY/tree with 100 GFW (r = 0.997) suggests that loss in total yield is due to reduced fruit weight (Table 1).

Table 1 : Effect of bacterial leaf spot intensity and green fruit yield in almond cv. Merced

Leaf spot Intensity (%)	Gummosis Intensity (%)	Per cent in loss in	
		Green fruit 100 green	Yield tree fruit weight
1.27	2.00	1.22	5.37
10.50	13.00	15.99	16.15
24.67	33.33	32.50	29.02
42.33	61.33	49.80	42.82
61.00	91.66	69.54	58.85

Effect of gummosis severity :

Gummosis severity significantly reduced the 100 GFW and 100 FNW of all the 3 cultivars and the reduction being directly proportional to the increasing disease level. Regression coefficients of GFW and FNW vs GS were highly significant and not homogenous in the 3 cultivars tested (Fig 1 & 2).

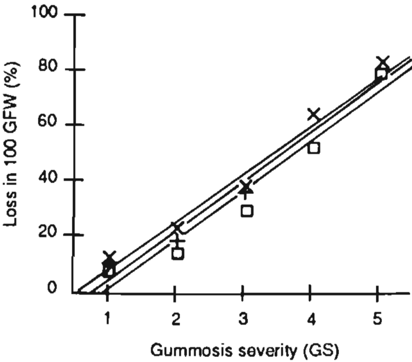

Fig. 1 Estimated linear relationship between loss in 100 GFW & GS

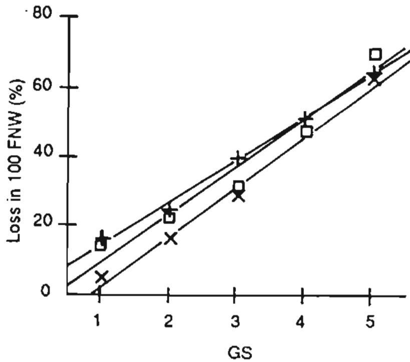

Fig. 2 Estimated linear relationship between loss in 100 FNW 1 GS

The simple linear correlation between loss in GFW and FNW with GS can be measured by a single coefficient of 0.984 and 0.994, respectively in various cultivars tested. The loss in 100 GFW is expected to fall between 18.04 $\pm$ 4.28, 18.11 $\pm$ 5.34 and 17.48 $\pm$ 3.17 per cent in cv. Afghanistan Seedling, Merced and Non Pareil, respectively at P = 0.05. The corresponding figures for FNW are 12.48 $\pm$ 0.77, 13.81 $\pm$ 2.65 and 14.33 $\pm$ 5.99, respectively.

It is clear from the present investigation that bacterial leaf spot and fruit gummosis of almond caused substantial losses in the fruit yield. The reduction is directly correlated with the increase in disease level. Proper control measures at an appropriate time will reduce the leaf and fruit infection and increased the fruit yield (JINDAL *et al.*, 1989).

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Pathogenic variation in *Xanthomonas campestris* pv. *undulosa*

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ABSTRACT

In the frame of a cooperative network on bacterial diseases of cereals set up by CIMMYT, Mexico, more than 240 strains of *Xanthomonas campestris* pv. *undulosa* were isolated from leaf streak and black chaff symptoms on bread and durum wheat as well as on triticale, from various African, American and Asian countries. Pathogenicity has been assessed under controlled environmental conditions by infiltration of a bacterial suspension into the leaves and/or by puncture of the plantlets with a contaminated needle. Clear differences in aggressiveness were noted among strains from various geographical origins. The typical pv. *undulosa* strains induced by puncture inoculation extensive leaf stripe symptoms with exudates on wheat and on barley. Strains isolated from some areas in Bolivia, Brazil, Ethiopia, Pakistan and South Africa induced only limited symptoms. This suggests occurrence of differences in the local populations of pv. *undulosa*. Variants with reduced aggressiveness were obtained from some typical pv. *undulosa* strains after storage at -28°C under glycerol. All typical pv. *undulosa* strains reacted with monoclonal antibody AB3-B6 raised against *X. c.* pv. *undulosa*, except strains with reduced aggressiveness. Strains of pv. *translucens* were clearly differentiated from pv. *undulosa* by their restricted pathogenicity to barley.

Keywords : bacterial leaf stripe, wheat, barley, serology.

INTRODUCTION

Bacterial leaf stripe of wheat (*Triticum aestivum* L.) caused by *Xanthomonas campestris* pv. *undulosa* (Smith, Jones and Reddy) Dye 1978, is characterized by elongated and water-soaked leaf streaks evolving into greasy areas with production of yellow exudates. It is known as black chaff when found on the glumes. Since its report from USA.in 1917, the bacteria has been detected in

most of the wheat growing countries in warm areas. Since 1980, the disease has been reported from China, Pakistan, Mexico, Brazil, Zambia and might be considered as the main bacterial disease on wheat, with regards to yield loss it causes (DUVEILLER *et al.*, 1991).

A considerable confusion actually exists concerning the pathovar naming due to the tendency to group under pv. *translucens* (Jones, Jonhson and Reddy) Dye 1978 strains differing in host range and thus in economic importance (BRADBURY, 1986). Besides variation in the host spectrum, differences in aggressiveness must be taken into account as for correct evaluation of the potential risk they represents, and for identification of highly aggressive strains for resistance screening of germplasm.

The purpose of this study is to analyse differences in aggressiveness within a collection of *X. c.* pv. *undulosa* strains from various geographical origins.

MATERIAL AND METHODS

Culture collection Neopathotype or reference strains of related pathovars were used as control, i.e. strain NCPPB1944 for *X.c.* pv. *cerealis*, NCPPB2700 for *X.c.* pv. *graminis*, NCPPB2389 for *X.c.* pv. *hordei*, NCPPB973 for *X.c.* pv. *translucens* , NCPPB1515 for pv. *oryzicola* and NCPPB1945, 2821, 2825, 2826, 2828 and CFBP3085 for pv. *undulosa*. Strains provided by J. Smith (South Africa) as *X. c.* pv. *translucens* (UPB674, 682), by J. Chipili (Zambia) and Y.R. Mehta (Brazil) as *X.c.* pv. *undulosa* (UPB543 and from 752 to 758, respectively) were also included. Typical single colonies appearing on nutrient agar supplemented with 5% glucose isolation plates, or reference cultures provided by donors were streaked out on GYS plates for purity checking. Standard bacteriological tests were applied for confirmation of identification as *X. campestris*.. Indirect immunofluorescence was performed on pure culture with the rat monoclonal antibody AB3-B6 at 44 µg/ml (BRAGARD *et al.*, 1992 ; DUVEILLER & BRAGARD, 1992). The origin of *X.c.* pv. *undulosa* strains is described in table 1. Subcultures were kept on Dye's GYCA (1962) slopes at 4°C in the dark, for up to 1 year, or lyophilised before long-term preservation in the same conditions. Glycerol suspensions were also stored at - 20°C.

Pathogenicity testing Seedlings of barley (cv. Corona), wheat (cv. Capitaine and Alondra) and oat (cv. Alfred) were grown up to the three-leaf stage in an air-conditioned greenhouse at 20-17°C, day/night temperatures, under a 14 h

Table 1. Origin of the *X. campestris* pv. *undulosa* strains

Origin		Year of isolation	Number of strains	Host plant
Africa	Ethiopia	1988	1	*T. aestivum*
	Madagascar	1991	5	*T. aestivum*
	South Africa		5	*T. aestivum*
			2	*T. durum*
	Tanzania	1987	5	*Triticum x Secale*
	Zambia	1986	2	*T. aestivum*
		1988	6	*T. aestivum*
America	Argentina	1988	18	*T. aestivum*
		1991	1	*T. aestivum*
	Bolivia	1988	3	*T. aestivum*
	Brazil	1988	9	*T. aestivum*
		1988	10	*Triticum x Secale*
		1990	65	*T. aestivum*
	Canada	1943	1	*T. aestivum*
		1969	1	
		1966	1	*T. durum*
		1968	2	*Triticum x Secale*
	Mexico	1987	35	*T. aestivum*
		1988	4	*T. aestivum*
		1987	12	*Triticum x Secale*
	Paraguay	1988	2	*T. aestivum*
	Peru	1989	1	*Triticum x Secale*
	USA	1988	6	*T. durum*
		1989	1	*T. durum*
	Uruguay	1987	15	*T. aestivum*
Asia	Pakistan	1988	5	*T. durum*
	Syria	1988	5	*T. durum*
		1989	2	*T. durum*
	Turkey	1987	5	*T. aestivum*
		1987	5	*T. durum*
		1988	5	*T. aestivum*
		1988	1	*T. durum*

photoperiod. Ten plants per cultivar and per strain were inoculated. Strain NCPPB2821 was used as positive control with strain CFBP3085 (UPB513) isolated from triticale at El Batan, Mexico, for *X.c.* pv. *undulosa.* Strains NCPPB973 for *X.c.* pv. *translucens* and NCPPB2389 for *X.c.* pv. *hordei* were also included.

The basic test for pathogenicity assessment is the pricking inoculation (COLIN *et al.*, 1990). Plants were puncture inoculated with a sterile needle passed through 24h colonies, after injection of sterile water into the leaf sheaths at 2.5 cm above the soil level. For infiltration injections, a 2-ml disposable syringe (B. Braun, Melsungen, W-Germany) was fitted with a 5 mm piece of polyethylene tubing. By means of this device, the bacterial suspensions were infiltrated through the upper leaf surface, at 5 cm from the tip, by pressing between a finger and the end of the

tube, until appearance of a water-soaked area of about 2 cm length. Plants were incubated for 10 days or more at 24-17°C day-night temperatures in a dew chamber.

RESULTS AND DISCUSSION

The reference *X. c.* pv. *undulosa* strains induced by pricking inoculation on wheat cv. Capitaine and Alondra water-soaked areas at day 3, evolving into translucent spots or streaks showing a greasy apearance and exudates after 5 to 7 days. Lesions turned yellow to light brown and leaf tissues were macerated at day 10. Lesions generally extend more than 5 cm around the inoculation point. Symptoms on barley cv. Corona were similar to those observed on wheat, but exudate production was often delayed or restricted. The lesions are extending slightly less and are often bordered by a yellow halo. On oat cv. Alfred, the strains induced generally only light yellow streaks on the leaf (Table 2).

By infiltration inoculation, symptoms were the same, except that lesions extension was limited to the infiltrated area. Most of the other strains isolated from *Triticum aestivum*, *T. durum* or *Triticum x Secale* behaved similarly to the reference strain. Weak aggressive strains, however, have been isolated from several bread and durum wheat samples both from nurseries and farmer's fields collected in Bolivia, Brazil, Ethiopia, Pakistan and South Africa. When seedlings were puncture inoculated, lesions were readily formed but with only limited extension. They produced on wheat small water-soaked areas, sometimes with exudates, but never extending more than 25 mm around the inoculation point and usually limited at 5 mm. Symptoms on barley were limited by a necrotic border, no exudation was noticed, but the yellowing was extending up to 10 mm. Lesions on oat were also limited in extension, but often appeared water-soaked and turned red-brown after 7 days. Nevertheless, symptoms on leaves from which these strains were isolated were similar to those from which the typical strains had been obtained. Strains isolated from rye leaves collected in Mexico and USA and which could be considered *X. c.* pv. *secalis* according to Bradbury (1986), induced symptoms similar to those of the deviating *X.c.* pv. *undulosa* strains. However, they differ from our *X. c.* pv. *secalis* reference strain in their serological reaction (unpublished data). Variants inducing also limited symptoms have been detected on strains kept under glycerol at -20°C or after long-term storage at -80°C. Pathogenicity of lyophilised cultures was not altered, even after 5 years of storage.

By infiltration inoculation, strains with altered aggressiveness, such as strain

Table 2 : Reaction of *Xanthomonas campestris* pv. *undulosa* strains by pricking inoculation on barley cv. Corona, oat cv. Alfred, wheat cv. Alondra and in indirect immunofluorescence (IF), compared to reference strains.

Collection Number	Origin		Disease severity[a]			IF[b]
	country	plant	barley	oat	wheat	
	Africa					
UPB664	Ethiopia	*Triticum aestivum*	3N	1-2	3N(B)	-
UPB727		*Triticum x Secale*	5*	1	5*(C)	+
UPB876	Malagasy	*T. aestivum*	5*	1	5*(C)	+
UPB674	South Africa	*T. aestivum*	5*	1	5*(C)	+
UPB682		*T. durum*	1N	1-2	3N(A)	-
UPB615	Tanzania	*Triticum x Secale*	5*	1	5*(C)	+
UPB543	Zambia	*T. aestivum*	5*	1	5*(C)	+
UPB644			5*	1	5*(C)	+
	America					
UPB424	Argentina	*T. aestivum*	4*	1	4*(B)	+
UPB663	Bolivia	*T. aestivum*	5*	1	5*(C)	+
UPB670			1N	1-2	3(B)	-
UPB605	Brazil	*T. aestivum*	1N	1-2	3(A)	-
UPB606			5*	1	5*(C)	+
UPB752			4*	1	4*(B)	+
UPB753			5*	1	5*(C)	+
UPB754			1N	1-2	3N(A)	-
UPB755			1N	1-2	3(A)	-
UPB756			1N	1-2	3(A)	-
UPB757			5*	1	5*(C)	+
UPB758			1N	1-2	4*N(B)	-
UPB445	Canada		4*	1	5*(C)	+
UPB487	USA	*T. durum*	4*	1	5*(C)	+
UPB496			4*	1	5*(C)	+
UPB513	Mexico	*Triticum x Secale*	5	1	5*(C)	+
UPB513variant			2*	1	4(A)	-
UPB522			4*	1	5*(C)	+
UPB570		*T. aestivum*	4*	1	5*(C)	+
UPB594			4*	1	5*(C)	+
UPB658			4*	1	5*(C)	+
UPB650	Paraguay	*T. aestivum*	4*	1	5*(C)	+
UPB733	Peru	*Triticum x Secale*	4*	1	5*(C)	+
	Asia					
UPB480	Pakistan	*T. durum*	4	1	3N(B)	-
UPB482			4*	1	5*(C)	+
UPB645	Syria	*T. durum*	5*	1	5*(C)	+
UPB352	Turkey	*T. aestivum*	4*	1	5*(C)	+
UPB661			5*	1	5*(C)	+
Reference strains						
pv. *cerealis*	USA	*Bromus inermis*	4*	1	5*(C)	+
pv. *hordei*	India	*Hordeum vulgare*	5	1	1(B)	+
pv. *graminis*	Switzerland	*Dactylis glomerata*	1	1	1(A)	-
pv. *oryzicola*	Malaysia	*Oryza sativa*	0-1	0-1	0-1	-
pv. *translucens*	USA	*H. vulgare*	5	1	1(B)	+
pv. *undulosa*	Canada	*T. aestivum*	4*	1	5*(C)	+

[a]Disease severity rated 10 days after inoculation : 0, no symptoms ; 1, yellowing ; 2, water-soaked areas ; 3, greasy aspect ; 4, translucence ; 5, maceration and collapse of the inoculated tissues ; * = exudates, N = necrosis. Extension of lesions A = 0.1-10 mm, B = 10 - 50 mm, C = more than 50 mm around the inocultation point.

[b]+ = positive reaction, - = negative reaction.

UPB670, produced on wheat a green water-soaking, but no exudates and often an early collapse. Infiltrated area became yellow during tissue collapse and surrounded by a brown border on barley cv. 'Corona'. On oat , the lesions first appeared slight brown and later on red-brown with tissue maceration.

The altered aggressiveness is related to a non-reaction in indirect immunofluorescence and ELISA (data not shown) with the monoclonal antibody AB3-B6. This antibody is reacting with a polysaccharidic compound of *X.c.* pv. *cerealis*, *hordei*, *translucens* and *undulosa*, but neither with pv. *graminis* nor with pv. *oryzicola*.

The difficulty of differentiating between *X.c.* pv. *cerealis* and pv. *undulosa* or pv. *secalis* is reflected in this study. Reference strains react indeed exactly in the same manner on the used barley, oat, wheat cultivars. Studies on a wider host range are in progress

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Light and transmission electron microscopical study of the interactions between *Xanthomonas campestris* pv. *translucens* and pv. *undulosa* on wheat, barley and oat

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ABSTRACT

Leaves of wheat (cv. Alondra), barley (cv. Corona) and oat (cv. Alfred) plantlets were inoculated by infiltration of 10^6 cells/ml of *X.c* . pv. *translucens* (NCPPB 2389), *X.c.* pv. *undulosa* (UPB 513) and a deviating pv. *undulosa* strain (UPB 670). The compatible interactions including the strains NCPPB 2389 on barley and UPB 513 on barley and wheat were characterized by the filling of the whole intercellular spaces with bacteria embedded in a background matrix together with microfibrils. The origin and nature of the microfibrils, frequently associated to a plant cell wall shredding, is discussed. In the incompatible interactions of these strains on oat, no symptom, nor bacterial multiplication could be detected in the tissues. With strain UPB 670 on the wheat, rapid cell collapse occurred, bacteria were found in the intercellular spaces but the fibrillar matrix was strongly reduced and plant cell wall shredding was not detected. On barley, few distorted bacteria were found encapsulated by a narrow film at the plant cell surface, whereas on oat, bacterial multiplication and cell collapse were delayed.

INTRODUCTION

The bacterial leaf stripe disease of small grain cereals, caused by various pathovars of *Xanthomonas campestris* Dowson 1939, is characterized by small linear water-soaked spots or streaks on the lamina, often covered by small yellowish exudates. The distinction between the pathovars is based on the host range of the strains; *X.c.* pv. *undulosa* (Smith, Jones & Reddy) Dye 1978, being pathogenic on wheat, barley and sometimes also on oat, while *X.c.* pv. *translucens* (Jones, Johnson & Reddy) Dye 1978, is restricted to barley (BRADBURY, 1986).

During a study of pathogenic variations in a collection of *X.c.* pv. *undulosa* strains isolated from wheat samples of various origins, an altered pathogenicity to wheat, barley and oat was noticed for some strains (BRAGARD & MARAITE, 1992). In order to better understand the mechanisms underlaying the differences in strain host range and aggressiveness, light and transmission electron microscopical (TEM) studies were done on interactions of *X.c.* pv. *translucens* and *X.c.* pv. *undulosa* strains in these plants.

MATERIALS AND METHODS

Plant material and inoculation. Plantlets of wheat (cv. Alondra), barley (cv. Corona) and oat (cv. Alfred) were grown in a greenhouse up to the three-leaf stage. One day before the inoculation, they were transferred to a growth room at 17/25°C (night/day), 70-100%R.H., and a 12h photoperiod.

Inoculum preparation and inoculation. The strains *X.c* pv. *translucens* NCPPB 2389, *X.c.* .pv. *undulosa* UPB 513 and the deviating strain *X.c.* pv. *undulosa* UPB 670, were choosen on base of their differential reactions on barley, wheat and oat after leaf sheath inoculation by pricking. Symptoms description is given elsewhere (BRAGARD & MARAITE, 1992). A bacterial suspension of 10^6 cells/ml was prepared and infiltrated into the leaves as described by these authors. After inoculation, the plants were immediately replaced in the humide chamber. Symptoms evolution was followed during 12 days and samples taken for microscopical analyses 4h, 1, 2, 5, 6, 7 and 9 days after inoculation.

Samples preparation for light and electron microscopy. Leaf samples were prepared following the classical double fixation procedure by successive immersion in 4% (v/v) glutaraldehyde and 2% (v/v) osmium tetroxyde in 0 .1 M sodium cacodylate buffer (pH 7.2) supplemented with 0.1% ruthenium red, dehydratation in ethanol solutions and embedding in Spurr resin. For light microscopy, sections were stained in 0,1% toluidine blue in 1% (w/v) aqueous borate solution. Ultrathin sections were stained with 2% aqueous uranyl acetate and Reynold's lead citrate.

RESULTS AND DISCUSSION

In the compatible interactions of NCPPB 2389 on barley and of UPB 513 on wheat and barley, the infiltrated area became water-soaked and exudates appeared on the lamina between day 5 and day 7 after the inoculation.

Collapse of the tissues occured only on wheat at day 12. Only limited plant cell wall distortion and plasmalemma retraction were noticed in the water-soaked lesions between day 5 and day 7, whereas numerous bacteria, embedded in a toluidine blue coloured matrix, occupied the whole intercellular spaces (Fig. 1). This matrix appeared to contain a fibrillar material (Fig. 2) similar to that described for various other compatible interactions with xanthomonads (MORGHAM *et al.*, 1988; BRETSCHNEIDER *et al.*, 1989), pseudomonads (SMITH & MANSFIELD, 1982; BROWN & MANSFIELD, 1988) and clavibacter (BENHAMOU, 1991). The origin and nature of such a fibrillar matrix has been considered either as plant cell wall alteration products (WALLIS *et al.*, 1973; CASON *et al.*, 1978; BENHAMOU, 1991), either as bacterial exopolysaccharides (AL-MOUSAWI *et al.*, 1982; JONES & FETT, 1985), or as both together (BROWN & MANSFIELD, 1988). Our observations suggest that the fibrils are products resulting probably from the recent degradation of the plant cell wall by the bacterial enzymatic activity and that the background matrix, in which the fibrils accumulate, is mainly composed of bacterial exopolysaccharides mixed with digested cell wall constituents. Indeed the fibrils are frequently associated to a shredding of the pectin-rich outer part of the cell wall (Fig. 2 and Fig. 3) and are not visible in absence of ruthenium red. Presence of a background matrix is demonstrated by its visible retraction in some intercellular corners, possibly during the fixation process. In several barley leaf samples, bacteria were locally viewed disposed perpendicularly to the plant cell surface (Fig. 1). This suggest that bacteria are able to move in the fibrillar matrix, attracted by something either initially present at the cell surface or diffusing through the walls. In the incompatible interactions of NCPPB 2389 on wheat, symptoms were limited to small yellow spots on the lamina. Only restricted plant cell alteration, coloured matrix accumulation and bacterial multiplication were detected in small areas of the lesions. No symptom appeared in the interactions of these strains on oat and no bacteria could be seen by TEM. The inoculum concentration choosen to obtain a progressive development of the disease, however was to low to allow visualization of the events occurring at the plant-bacteria interface in the first hours of all the interactions, particularly in the incompatible ones. Higher inoculum concentrations will be used in further studies.

At day 5 after the inoculation, UPB 670 induced a green water-soaking on wheat. Intense plant cell wall distortion and plasmalemma retraction already occurred at day 5 in most of the cells and was followed by a general collapse of

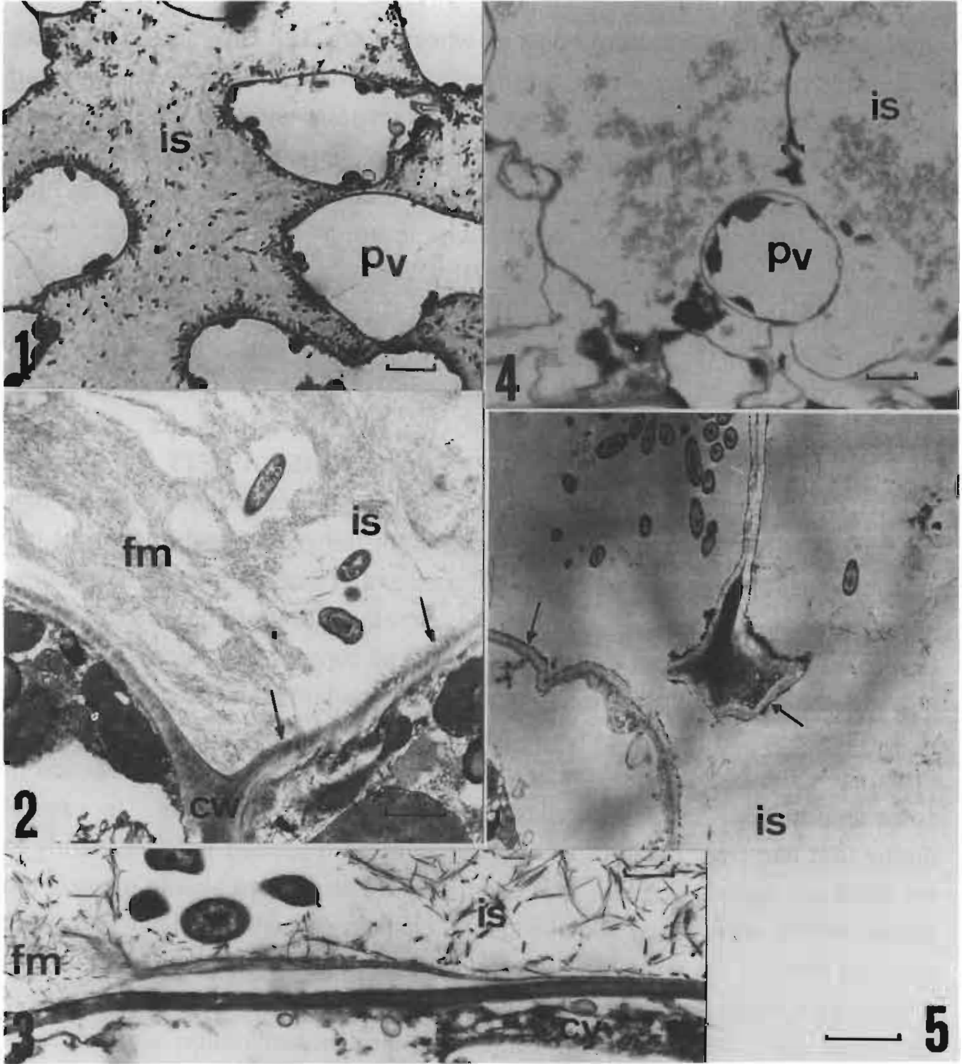

FIGURE 1 : Barley inoculated with UPB 513, 6 days after the inoculation. Intercellular spaces are filled by a toluidine blue matrix and numerous bacteria. (Bar=10µm).
FIGURE 2 : Wheat inoculated with UPB 513 at day 5. A fibrillar material similar to that associated to the plant cell wall shredding (arrows) accumulates in the intercellular spaces between the bacteria. (Bar=1µm).
FIGURE 3 : In the same interaction, shredding of a part of the outer plant cell wall liberates fibrils similar to those seen in the matrix. (Bar=0,5µm).
FIGURE 4 : Wheat inoculated with UPB 670 at day 6. Intense cell collapse and bacterial aggregates randomly dispersed in the intercellular spaces without any blue matrix. (Bar=10µm).
FIGURE 5 : In the same interaction, reduced amount of fibrils between the bacteria and no apparent cell wall shredding. A dark granular material covers the outer part of the plant cell walls (arrows). (Bar=2µm).

Abbreviations : CW= cell wall; CY=cytoplasm; FM=fibrillar material; IS=intercellular space; PV=plant vacuolar content.

the cells at day 7 (Fig. 4). Bacteria were found distributed in aggregates randomly dispersed through the intercellular spaces but not surrounded by a toluidine blue coloured matrix. TEM revealed the presence of very limited amount of fibrils near the bacteria and absence of shredding on the outer part of the plant cell wall (Fig. 5). The walls appeared covered by a dark granular material also present in the middle lamella and which probably results from an alteration of the cell wall pectic compounds by an enzymatic activity differing from that occurring in the interactions with UPB 513. The absence of matrix and the bacterial aggregation could be considered as artefacts of the fixation as it may be suspected in such a process. Indeed, a difference of composition between the exopolysaccharidic slime of this deviating strain and the two other ones, has been detected during immunological studies (BRAGARD & MARAITE, 1992). This could be correlated with a difference of sensitivity to the solvents implicated in the preparation of the samples. On barley, UPB 670 induced an atypical reaction limited to a yellowing of the lamina without any water-soaking. A reduced number of bacteria distorted by vesicular wall evaginations were found encapsulated on the plant cell surface in a light film without fibril. A similar encapsulation has already been mentionned for incompatible interactions with other bacteria (SMITH & MANSFIELD, 1982; MORGHAM *et al.*, 1988; BROWN & MANSFIELD, 1988). This phenomena is generally considered as an active process of immobilization and inactivation of the pathogen by the host cell. Absence of plasmalemma retraction or cell collapse is in accordance with this hypothesis. On oat, the bacteria of the UPB 670 stain, although distorted and encapsulated at the plant cell surface as in barley, reached detectable levels only at day 7 and induced cell collapse, contrarily to the typical pv. *translucens* and pv. *undulosa* strains.

Further studies, with higher inoculum concentration, will be performed to visualize and understand the events occurring at the plant-pathogen interface in the first hours after the inoculation. These events must be determinant to explain the differences recorded in the present work.

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Relationship between pathogenicity and epiphytic fitness of Tn5 mutants of *P.s.* pv. *syringae* on pear

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ABSTRACT

A procedure based on the specific effect of relative humidity (RH) has been selected to check the epiphytic fitness of strains of *Pseudomonas syringae* pathovar *syringae*. After spraying with a bacterial suspension the plants were placed in a growth chamber and kept (i) for two days under 70 % of relative humidity (RH), (ii) then for seven days under 100 % of RH and finally (iii) under 40 % of RH for the last eight days. This procedure was performed to compare epiphytic fitness of non pathogenic Tn5 mutants of *P. s.* pv. *syringae* to that of the wild type strain on pear leaves. During the first step (70 % RH), the populations of both wild type strain and non pathogenic mutants decreased, but the populations of mutants reached a lower level than those of the wild type strain. During the second step (100 % RH), a bacterial multiplication occured in all cases. Then during the last step (40 % RH), while the populations of the wild type strain were decreasing slowly, those of non pathogenic mutants decreased fastly. Furthermore, the frequency of leaves with epiphytic populations was lower for non pathogenic mutants than for the wild type strain.

We concluded that the ability of non pathogenic mutants to multiply on pear leaves was not affected when high relative humidity was supplied. However, these mutants showed a weak ability to survive under dry conditions.

KEY-WORDS : *Pseudomonas syringae* pathovar *syringae*, epiphytic fitness, *Pyrus communis*, Tn5, *hrp, dsp* genes

Table 1 - Bacterial strains used in this study

Type of bacteria	number	phenotype
wild type	2027-37	Pat^+, HR^+
Tn5 mutants	PS 45-25	Pat^+, HR^+
	PS 59-35	Pat^+, HR^+, INA^-
	PS 29-36 PS 45-48	$Pat^{+/-}$, HR^+ (Vir)
	PS 56-34 PS 75-42	Pat^-, HR^+ (Dsp)
	PS 76-37	Pat^-, HR^+ His^-(Dsp)
	PS 88-1	Pat^-, HR^- (Hrp)

Pat^+ : pathogenic on pear
HR^+ : induce a hypersensitive reaction on tobacco
INA^- : ice nucleation inactive
$Pat^{+/-}$: non pathogenic on pear leaf but causes necrotic reaction on immature pear fruit
Pat^- : non pathogenic on pear
His^- : auxotrophic mutant requiring histidine for growth.

INTRODUCTION

Pseudomonas syringae pathovar *syringae* (PSS) is a typical necrogenic plant pathogen. It has been frequently used as a model for the study of the plant/bacterium interaction during the infection process. All genetics traits involved in the establishment of the disease have been described for this bacterium. Besides, PSS have been found to occur in large numbers on the surface of healthy plants. This result has led to a widely accepted hypothesis that PSS is capable of epiphytic growth on healthy plants providing inoculum in absence of disease.

The aim of this work was to study the relationship between the pathogenicity and the ability of epiphytic survival of PSS in the case of the bacterial blast of pear.

MATERIAL AND METHODS

• *Bacteria*

The bacteria used in this study are listed in table 1. Mutants were obtained by Tn5 mutagenesis using the procedure described by LINDGREN *et al*, 1986. Non pathogenic mutants were selected using the detached leaf assay described by YESSAD *et al*, 1992.

• *Plants*

Pear seedlings were obtained from open pollinated *Pyrus communis* cv. "Fieudière". They were grown in a greenhouse from seeds after breakdown of the dormancy. They were transferred in growth chamber one week before inoculation.

• *Monitoring of bacteria*

Bacterial suspensions (1 x 10^6 cfu/ml) in sterile distilled water were sprayed on both faces of leaves until run off. Ten leaves from each subplot were collected two hours, then two, seven, nine and seventeen days after inoculation. Each leaf was washed individualy in 4.5 ml of sterile distilled water by vortexing for 1 min. Fifty microliter of ten fold dilution series were plated onto King'B medium supplemented with kanamycin (30 µg/ml) when required. Colonies were enumerated after incubation at 25°C for 72 hours.

fig 1 : dynamics of *P.s.* pv *syringae* mutants on leaf surface of pear seedling

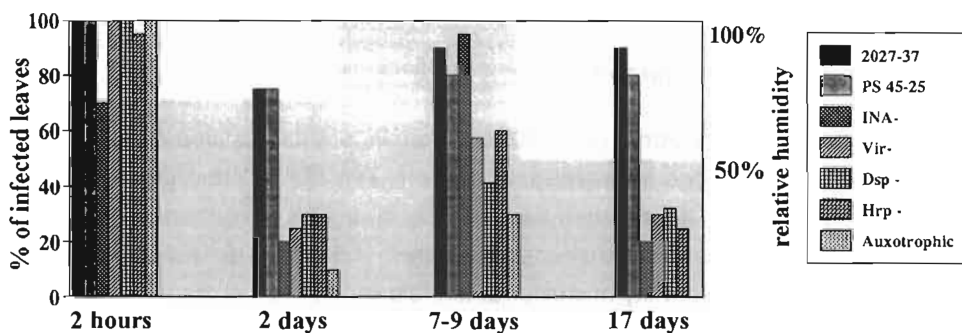

fig 2 : frequencies of infected leaves after inoculations

• ***Environmental conditions***

The plants were kept in an environment controlled chamber at the température of 20°C ; three different relative humidity levels were applied successively to the plants : 70 % for two days, 100 % for seven days and 40 % for the last eight days.

RESULTS

Two parameters were monitored to characterize the epiphytic fitness of bacteria : quantification of bacterial cells on leaf surfaces (fig. 1) and frequency of contaminated leaves estimated at the end of each different RH period (fig. 2).

During the first period (RH = 70 % for two days) the populatioins of both wild type strain and non-pathogenic mutants decreased. However, the dynamics of the non-pathogenic mutants appear much more affected than that of the parental strain or the weakly altered mutant as well (PS 45-25).

During the second period (RH = 100 % for seven days) the multiplication of bacterial populations occured in all cases excepted for the case of the auxotrophic mutant (PS 76-37).

During the last period (RH = 40 % for eight days) while the populations of the parental strain and that of the weakley pathogenic mutant (PS 45-25) were decreasing at a rather week rate, those of other mutants decreased quicker and deeper whatever the type of phenotype they expressed (altered virulence, Dsp, Hrp or INA^-).

The analysis of the frequencies of infected leaves at the end of each period (fig. 2) confirmed the analysis *i.e.* low relative humidity in the growth chamber led to a more important decrease of the frequency of infected leaves of plants contaminated with either non-pathogenic bacteria or INA^- mutant. At the opposite, high humidity in the growth chamber favoured a rather successfull colonisation of leaves.

CONCLUSION

As previously shown by numerous authors, colonization and multiplication of bacteria either pathogenic or saprophytic on the leaf surface of plants appeared to be influenced by environmental conditions mainly by humidity. However, when the bacterial functions playing a role in the plant infection

processes are altered (Hrp$^-$, Dsp$^-$, altered virulence) the ability of such strains to survive on the leaf surface is strongly affected when placed under non-favorable environmental conditions.

The lack of ability for the INA$^-$/Pat$^+$ mutant to survive on the leaf surface under unfavorable conditions suggests that the protein involved in the ice nucleation activity which is located in the outer membrane of the bacterium could play a role in the interaction with plant at the leaf surface level.

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Shoot susceptibility to fire blight (*Erwinia amylovora*) of Asian pear cultivars

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ABSTRACT

The introduction of Asian pear in Europe is recent. Very few experimental or field information is available on fire blight susceptibility of these pears, which are originally grown in areas where it is not present.

In order to test fire blight susceptibility, a collection of 24 cultivars of Asian Pear, selected for their agronomic interest by B. THIBAULT was planted in the experimental field of DAX (INRA-EEC-SPV) within the framework of an EEC programme on fire blight control. The trees were shoot-inoculated during the growth period in May (89, 90 and 91) with a strain of *E. amylovora* (CFBP 1430), under field conditions.

Results showed that, with the exception of the cultivar "Shinko", most of the Nashi cultivars, introduced in Europe, are susceptible to fire blight on shoots. In contrast, the tested "Li" cultivars seemed to be less susceptible. But their number in our experiment was low. These results should be confirmed by observation in commercial orchards, under natural infection.

KEY-WORDS : Asian pear, *E. amylovora*, fire blight , Li, Nashi

INTRODUCTION

Asian pears have been grown in China before the beginning of the Christian era, but these fruit-trees were particularly studied and selected in Japan during the current century. In Europe, some plantings of introduced or nursed trees were recently attempted as alternative crops. The overall level of production is still very low.

These pears belong to different species ; among them, *Pyrus pyrifolia* (otherwise called *Pyrus serotina*) gathers most of grown cultivars known under the common name of "Nashi". The other cultivars called "Li" mainly belong to *Pyrus ussuriensis* or *Pyrus brestschneideri* species (THIBAULT, 1985).

A few plants identified as *Pyrus serotina* or as *Pyrus ussuriensis* and known to be resistant to fire blight were already used in France either in breeding programmes (THIBAULT & MAAS GEESTERANUS, 1984) , or in susceptibility studies (THIBAULT *et al*, 1987). But very few (if any) experimental information is available on fire blight susceptibility of asian pears cultivars introduced in Europe. These pears were originally grown in areas where the disease is not present : therefore information on their field resistance is not available either.

PLANT MATERIAL

A collection was planted in 1981 in Angers and 25 cultivars were compared and assessed for their agronomic abilities under european conditions (THIBAULT, 1985). In order to test fire blight susceptibility, most of these cultivars (23 cultivars for fruit-production and one cultivar of *Pyrus serotina* used as rootstock) were propagated and planted in the experimental field of Dax (INRA-EEC-SPV) in the South West of France, within the framework of an EEC programme on fire blight control.

METHODS

When the trees were 3 years old, they were shoot-inoculated in the field during the growth period, in May, for 3 successive years (1989 to 1991) with the strain CFBP 1430 of *E. amylovora*. The number of successful inoculations, the length of necrosis and the total shoot length were measured 8 weeks after inoculation. A minimum of 20 shoots was usually inoculated for each cultivar. The mean number of inoculated shoots per cultivar is 45 for the whole experiment.

A method of classification previously used for the assessment of the susceptibility of pear varieties (THIBAULT *et al*, 1987) and which is derived from LE LEZEC and PAULIN (1984) was used for the rating of these 24 cultivars. This method was based on two criteria :

• the severity (S), mean of percentages of lesion lenght to the total length of the shoot, for blighted shoots.
• the frequency of infection (F), ratio between the number of blighted shoots and the total number of inoculated shoots.

A combination of these criteria gave an index of susceptibility (I) and the whole data made possible the rating of the cultivars into five classes of susceptibility.

RESULTS - DISCUSSION

As summarized in Table 1 (complete data will be published elsewhere), results showed that, except for the cultivar "Shinko", most Nashi cultivars introduced in Europe, were susceptible to fire blight on shoots. In contrast, the tested "Li" cultivars seemed to be less susceptible. But their number in our experiment was low.

Table 1 - Susceptibility on shoots of Asian pears according to the index of susceptibility (I)

Very low susceptible	Low susceptible	Moderately susceptible	Susceptible	Very susceptible	
Shinko	Jing Bai Li	Shinsui*	Hakko	Chojuro	Shinseiki
	Xue Hua Li	Kimizuka wase	Hosui	Yakumo	Choju*
		Ya Li	Okusankichi*	Kosui	Kikusui
		Pin Guo Li	Imamura Aki	Tama	*Pyrus serotina*
			Niitaka	Hayatama	Kumoi
				Suisei	Nijisseiki

Cultivars of Li are underlined
* 20 shoots only, inoculated from 1989 to 1991

Nevertheless *Pyrus serotina* appeared to be a species within which differences of susceptibility to fire blight exist between cultivars. Moreover, these results must be considered with caution : they provide information about what could be called a "potential susceptibility" of these cultivars. Several reasons suggest this remark :

- when tested, the trees were young and vigorous,
- the plants were inoculated during the full growth period in early spring, on succulent shoots,
- the environment and weather conditions were generally favourable for disease expression,
- under natural conditions of infection, we have not noticed many infections on the most susceptible cultivars, as we have seen on certain cultivars of *Pyrus communis*, under the same conditions,
- asian pears do not produce secondary blossoms.

Hence, the present report probably tends to overestimate the susceptibility of these Asian pears. Other assessment methods and blossom susceptibility of the same cultivars are currently under test, but the information presently available on the susceptibility of shoots is probably enough to discourage the planting of some of the cultivars of Nashi in certain areas.

RESUME

L'introduction de poiriers d'origine asiatique en Europe est récente. Il n'existe que très peu d'informations sur la sensibilité au feu bactérien de ces plantes, principalement cultivées dans des zones où la maladie n'est pas présente.

En 1981, une collection de 25 cultivars de Nashi et de Li a été plantée à Angers par B. THIBAULT pour étudier leurs qualités agronomiques. 24 d'entre eux ont été ensuite introduits au verger expérimental de Dax (INRA-CEE-SPV) dans le cadre d'un programme d'étude du feu bactérien soutenu par la CEE.

Les pousses des arbres ont été inoculées en période de croissance et en conditions de verger pendant trois années consécutives de 1989 à 1991 avec la souche CFBP 1430 d'*Erwinia amylovora*.

Les résultats montrent, qu'à l'exception du cultivar "Shinko", la plupart des cultivars étudiés de Nashi sont sensibles au feu bactérien sur pousses. Les quelques cultivars testés de Li sont par contre moins sensibles.

Ces résultats qui, compte-tenu des conditions expérimentales très favorables au développement de la maladie, tendent peut-être à surestimer le comportement de ces variétés, méritent d'être confirmés en conditions naturelles.

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Characterization of epiphytic *Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* pv. *vesicatoria* from symptomless weeds in Bulgaria

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Many plant pathogenic bacteria grow on leaf surfaces of healthy plants without casual visible symptoms (11,12). Several species of these microorganisms have a resident stage either on their hosts, or on non-hosts, in which they multiply and constitute the most likely source of inoculum for disease initiation on crops (7,11,12). Weeds, either as host or non-hosts, have been shown to serve as reservoirs of bacterial pathogens (2,3,9,13).

Some bacterial pathogens can colonize tomato (Lycopersicon esculentum) and multiply as foliar epiphytes before disease outbreaks (4,14).

Bacterial speck and bacterial spot of tomato, caused by Pseudomonas syringae pv. tomato (Pst) and Xanthomonas campestris pv. vesicatoria (Xcv), respectively, are sources of economic losses in tomato industry in Bulgaria. Pst and Xcv can be present as epiphytes on tomato leaves (4,14), seeds (6,9,16) and weeds (2,3,9,13).

The purpose of this study was:

1) To evaluate the symptomless development of Pst and Xcv on the common weed association on tomatoes, as well as their role as source of infection.

2) To characterize the natural epiphytic population of Pst and Xcv.

Materials and methods

Weed sampling and bacterial isolation

During the sampling period (1989-1991) 22 weed species were collected at random from both inside and outside (100 m from the borders) of bacterial speck and spot diseased fields of tomato in V. Tarnovo (North Bulgaria), Sofia (West Bulgaria)

and Plovdiv (South Bulgaria). These fields showed a moderate incidence of both bacterial speck and bacterial spot, ranging from 45-60% of the plants with symptoms.

Water filtrations from the roots and the overground part of every weed type (5 plants each) were performed using the Bogatzevska's method (1) for Pst and the method of Jones et al. (9) for Xcv. The presence of Pst and Xcv in the filtrates was demonstrated by inoculation on leaves of the test-plants (Chico and Ideal). Test-plants infiltrated with sterile water and pure culture of bacteria were used as controls (for Pst - 10^4 cfu/ml; for Xcv - 10^8 cfu/ml). Isolations were made from each type of weed and bacterium from the test-plants on King is medium B (KBM) for Pst and XTS and on XCV for Xcv (15).

Pathogenicity tests

The pathogenic properties of the reisolations from single spots on the test-plants were checked with:

1) Hypersensitive reaction on tobacco - HR (10)

2) Inoculation of tomato species Chico and Ideal using the vacuum infiltration method (1)

Biochemical characterization

The phenotype characteristic of the bacterium were determined according to the basic discriminative tests of identification of pv. from Ps. syringae (5,15) and X. campestris (5,15) (including determination of the pectolytic activity in CVP medium (15) and *Xanthan* synthesis (8)).

Results

Epiphytic survival on weeds

During the period of study the filtrates of 22 weed types from 16 crops sowed with tomato were analyzed. The filtrate of the following weeds did not cause a disease on the test-plants: Convolvulus arvensis, Sanquisorba minor, Amaranthus blifoides Setaria sp., Cirsium arvense, Sanchus arvensis, Chicorium intybus.

Bacteria were detected only from symptomless weeds collected inside the field of tomato showing bacterial speck and spot symptoms. Xcv was present epiphytically on the weeds: Amaranthus retroflexus, Solanum nigrum, Datura stramonium, Portulaca oleraceae, Abultilon avicennae, Chenopodium album, Sinapis arvensis, Galinzoga parviflora, Polygonum lapathifolium and Ranunculus repens.

The spots on the test plants (Ideal) were small, circular, brown without water-soaked zone. These symptoms are typical for bacterial spot and do not differ from these

of the Xcv control. The number of the spots on the test-plants varied between 15 and 100, depending on the density of the bacteria on the weed leaves.

Pst were recovered from: Amaranthus retroflexus, S. nigrum, D. stramonium, P. oleraceae, Andropogon halepensis, Echinocloa crus galli, A. avicennae, Cynodon dactylon, Xanthium strumarium, Agropyron repens.

The spots on the test-plant's leaves (Chico) were water-soaked with necrotic center and blighted zone, as well as elliptical brown spots on the stems, typical for the bacterial speck. The number of spots varied between 25 and 100 depending on the Pst density on the weeds.

Mixed populations of Xcv with Pst were found on the weeds: A. retroflexus, S. nigrum, D. stramonium and P. oleraceae.

Xcv and Pst were not found in the water extract of the weeds collected from healthy crops and uncultivated lands.

Characteristic of epiphytic populations and pathogenicity

The bacterial strains isolated from single spots of test-plants (111) were identified as Pst (60) and Xcv (51). Strains of Xcv from weeds were without pectolytic activity on CVP medium. The synthesized *Xanthan* did not show remarkable difference in the chemical formula.

All isolates (III) of the two pathogens were positive for HR on tobacco, Pst gives HR 18 hours after injection and Xcv after 24-36 hours.

All (60) Pst isolates produced water-soaked lesions with halos that first appeared 4-5 days after vacuum infiltration of 10^4 cfu/ml into leaf tissue. In 5-6 days after vacuum infiltration of 10^8 cfu/ml Xcv (51) formed small necrotic lesions without halos.

The weeds in the tomato crops can be divide into two groups depending on the epiphytical survival of Pst and Xcv:

1) Typical weed hosts - the bacteria are isolated as soon as the weed plants are collected near with tomato plants showing symptoms of bacterial speck and spot. They are a basic source of secondary infection. Typical examples are: A. retroflexus, S. nigrum, D. stramonium, P. oleraceae.

2) Non-typical weed host - the bacteria developed epiphyticaly mainly on the foliage not always on diseased plants:

- A. avicennae, G. parviflora, P. lapathifolium (for Xcv)
- S. dactylon, A. halepensis, E. crusgalli (for Pst)

3) Non-hosts - on which the bacteria never developed - C. arvensis, S. minor, A. blifoides, S. arvensis, Ch. intybus.

Pst and Xcv were not found in typical weed hosts of healthy tomato and uncultivated lands therefore this bacteria were not naturally disseminated.

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Fire blight of pear in Bulgaria

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Introduction

Fire blight caused by Erwinia amylovora (E.a.) (Burrill, Winslow et al.) was first observed in 1780 north of New York City. The disease was first noted in Europe in English pear orchards in 1957. In the late 1960 fire blight spread to the European continent by unknown means, where it inflicted considerable damages on pears, apples and ornamental trees and shrubs (Borecki et al., 1967; Beer et al., 1985; Psallidas and Dimova, 1986).

In 1990 fire blight was detected for the first time in Bulgaria.

The purpose of this study was to determine morphological, biochemical, serological characteristics and pathogenicity of the bacteria isolated from pear shoots showing symptoms characteristic of fire blight.

Materials and methods

Isolation

Isolations from diseased pear (variety Passe Crassane, Plovdiv areal) tissues were performed on King B medium (Schaad, 1988) using a method described by van der Zwet (1986). All cultures were maintained on potato sucrose agar (PSA). Bacterial suspension of every poor non fluorescent culture was plated on MS medium (Miller and Schroth, 1972); on D-3 (Kado and Heskett, 1970) and on high-sucrose medium (Crosse and Goodman, 1973).

Pathogenicity tests

The pathogenic ability of the isolates was tested:

-by infiltration on tobacco leaves for hypersensitive reaction - HR (Klement, 1963)

-by inoculation on green pear fruitlets (variety Bonne Louise) collected in early June (Schaad, 1988). Each isolate was tested in triplicate. Inoculated fruits were incubated in a moist chamber at 26°C, and the degree of necrosis and oozing was examined after 3-4 days. The test was repeated twice. Standard cultures of E.a. and Pseudomonas syringae were used as controls.

- on succulent pear shoots, using our own method: Young shoots (variety Bonne Louise) were vacuum infiltrated with a bacterial suspension (10^8 cfu/ml) from a 36 hours culture on PSA.

Identification of isolates

39 isolates and reisolates with a proved pathogenicity were identified using the standard bacterial methods (Dye, 1968; Schaad, 1988) according to Bergey's Manual of Systematic Bacteriology (1984).

Serological diagnostic

For the serological diagnostic of E.a. the method DAS-ELISA was used.

All reagent application parameters were optimised including determination of the optimal gamma-globulin fractions, conjugate and antigen concentrations. Different schemes of incubation and different types of conjugates were tested.

To determine the sensitivity of the method an antigen with 8 extract buffers prepared by fresh leaf of naturally infected pear was used.

The method was tested with 24 hours E.a. diluted from 10 to 10^8 cfu/ml in a physiological solution. Xcv was used for a control.

Results

Symptoms

On pear (variety Passe Crassane), in the orchards near Plovdiv, in early May a sudden necrosis and blackening of the blossoms and leaf were observed. The necrosis and blackening extended to the petioles of the leaf and blossoms and spread to the fruiting spur. The dead and blackened leaf and blossoms remained attached on the twigs. On the surface of the dead leaf and twigs under high humidity conditions a glistening mucoid substance was observed. Later in July and August dead twigs and shoots were observed in the trees as a result of the progress of the infection through the fruiting spurs to the shoots and twigs. Infected fruits turned black, shrunk and remained attached to the twigs. The observed symptoms on the different pear trees (variety Passe Crassane) were typical for fire blight, as described in the literature.

Isolation of the pathogen

All isolates made from infected plant tissues (shoots, dried fruitlets, twigs). None produced green or blue fluorescence pigments on KBM, formed characteristic orange-red colonies on the selective MS and D-3 medium. The bacterial colonies on HSM were shiny, opaque with small craters.

Pathogenicity

All isolates gave (4I) positive HR on tobacco leaf in 24 hours.

39 isolates induced characteristic necrotic spots on inoculated sites after 2-3 days on green pear fruitlets. From the center of this spots a milky white bacterial exudate came out.

18-24 hours after the vacuum infiltration the isolates (39) formed on the young pear shoots leaf black water-soaked spots typical for fire blight. Black spots with irregular form appeared on the stems of the fruitlets. The necrotic tissues died on the 3-4 day. 24 hours after the infiltration the blossoms were covered with brown water-soaked spots followed by tissue necrotization.

Our new developed method for inoculation is fast, accurate and easily applied during the whole vegetative period.

Identification

The main physiological and biochemical properties of the isolates (39) studied are shown in the Table 1.

Serological diagnostic

After the parameters were optimised and compared the most suitable to use with the fast and sensitive method DAS-ELISA were the following:

1) The 4 hours incubation of Ig6 under $37^{o}C$ and dilution with carbonate buffer pH 9.6 1:100

2) A 24 hours bacterial culture triple washed with a physiological solution (0.85% NaCl) and centrifuged 10 min. under 12000 g was used as antigen (10^8 cfu/ml). The antigen was incubated one night under $4^{o}C$. The use of fresh plant material showed that the most suitable dilution was 1:10 in PBS-T + 2% PVP + 0.2% OV.

3) It was given priority to an alkaline conjugate diluted 1:1000 in PBS-T + 2% PVP + 0.2% OV and incubated 4 hours under $37^{o}C$.

4) The substratum (p-nytrophenylphosphate) was incubated 1 hour under room temperature.

Under this scheme the measured extinction values (E) using bacterial cultures register differences between the diseased and healthy tests (3.7 to 4.6).

The reported differences of (E) between diseased and healthy fresh leaf is from 2.6 to 3.2.

Table 1

Characteristic of the isolates of E. amylovora from Bulgaria

Character		Bulgarian isolates	E.a.	Dye	Bergey's
Growth on MS		+	+	+	+
HR on tobacco		+	+	+	+
Pectate degradation		-	-	-	-
Hydrolysis of					
	gelatin	+	+	+	+
	aesculin	-	-	-	-
	starch	-	-	-	-
Production of					
	levan	+	+	+	+
	indole	-	-	-	-
H_2S from cystein		-	-	-	-
Nitrate reduction		-	-	-	-
Yellow pigment		-	-	-	-
Pink pigment on JDC		-	-	-	-
Motility		+	+	+	+
NaCl tolerance		4-5%	5%	3-6%	
Acid production from					
	L+ arabinose	+	+	+	+
	D- arabinose	v	-	-	-
	adonitol	-	-	-	-
	cellobiose	+	+	+	-
	dulcitol	-	-	-	-
	inulin	-	-	-	-
	lactose	-	-	-	-
	maltose	-	-	ND	-
	mannose	-	-	ND	-
	melibiose	+	+	+	+
	R+ raffinose	-	+/-	ND	-
	ribose	+	+	+	+
	salicin	v	-	v	v
	sorbitol	+	+	+	+
	sucrose	+	+	+	+
	trecholose	+	+	+	+
	xylose	+	+	+	-
Growth at 36°C		-	-	-	-

+ positive reaction; - negative reaction; v variable; ND not done

The sensitivity and specificity of the method DAS-ELISA give a possibility for fast and accurate diagnostic of a large number of tests.

The results obtained from the study of the physiological, biochemical, serological and pathological characteristic of the bacterial isolates obtained from diseased pear-variety Passe Crassane near Plovdiv indicated that the causal agent of disease was the bacterium Erwinia amylovora.

Fire blight was not detected on other pear varieties and orchards in the whole country.

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Advance of bacterial wilt of bananas in Mexico

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ABSTRACT

Bacterial wilt of bananas has been present and confined in Mexico only in the Tapachula and Villa Flores area of Chiapas state. In 1991 this disease appeared on a banana plantation in the Teapa region, state of Tabasco affecting some 20 plants. It is the first time that this Moko disease appears in the mexican gulf area. The causal bacterium is *Pseudomonas solanacearum*, race 2, biovar 1. A possible way of dissemination is discussed.

INTRODUCTION

Bacterial wilt of banana or Moko disease has been known since 1960 to cause serious problems in the Tapachula and Villa Flores regions of Chiapas that border on Guatemala (Buddenhagen, 1961, Santos, 1987). In October 1991, symptoms of this disease have been detected in an important banana region of Teapa, Tabasco. To confirm the suspected disease, an analysis of tissue samples was performed and a visit was made to the affected area.

MATERIAL AND METHODS

In October 1991, samples of different parts of diseased, five year old banana plants with bacterial wilt were received from a banana ranch of approximately 50 ha which borders on a tributary river and which, further down the stream, enters into the river Grijalva in the area of the city of Teapa, Tabasco. This tributary river, year from year, overflows its borders and inundates some zones of the mentioned ranch. Samples of corm, rachis, pseudostem and fruits were analyzed in order to isolate the

causal organism. Bacteria isolated from vascular system were purified and inoculated on young banana plants in the greenhouse. After symptom appearance, reisolations were made and the pathogenic isolates were then characterized in triplicate using the Hayward system (Hayward, 1988).

RESULTS AND DISCUSSION

From all the samples received, predominant one type of bacteria was isolated and purified. These isolates from all the samples on inoculation, produced wilting symptoms on young banana plants in the greenhouse after nine days. The reisolated bacteria on CPG (Casaminoacid-peptone-glucose) medium with tetrazolium chloride (Kelman 1954), produced typical fluidal and colored colonies and these were compared with the known *Pseudomonas solanacearum* isolates from Chiapas obtained from our collection.

The pathogenic bacterium is gram negative, oxidase and catalase positive, produces hypersensitive reaction in tobacco in 24 hours, does not rot potatoes and does not fluoresce, does not utilize maltose, lactose, cellobiose and sucros. It utilizes trehalose, but not mannitol, Sorbitol and dulcitol after 15 days. All isolates produced gas from nitrate, although some in more quantities than others. On the basis of this and its origin from banana, the bacterium was identified as *Pseudomonas solanacearum*, race 2, biovar 1. It is for the first time that this biovar is reported from Mexico on banana.

What is alarming, is the advance of this disease in Mexico (Fig.1), first reported in Chiapas in 1960 and now appearing in an important banana producing zone of Tabasco. Various factors may be responsible for its geographical advance, from Chiapas to Tabasco. It may be that the bacteria came with the high flooding river water, being carried from the wilderness area between Chiapas and Tabasco. Another possibility exists that the bacteria arrived by high winds and rain and insects from Chiapas or lake Izabal region in Guatemala, where it is known that this bacterium exists. This possibility is more remote, because of the distance involved. A

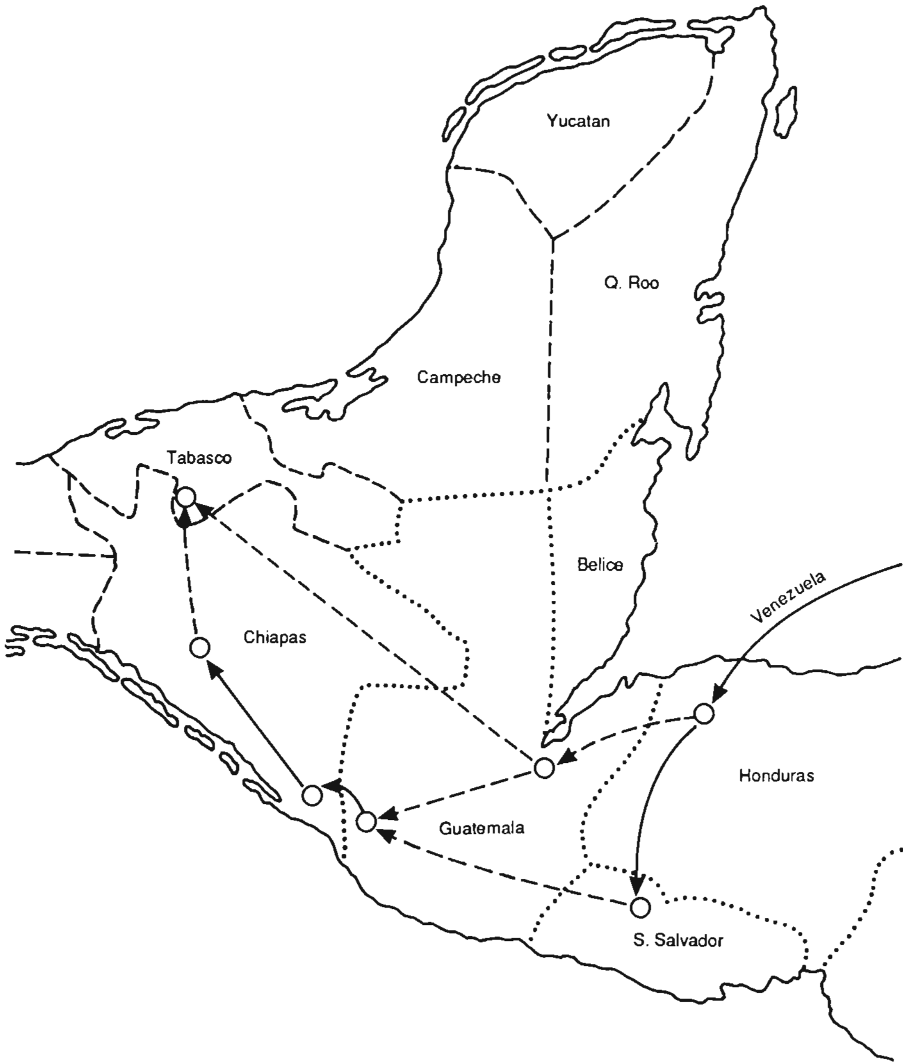

Fig.1. Advance of bacterial wilt of bananas

Advance of Moko on banana from South America to Honduras and San Salvador (after Stover, 1972), continuous line with arrow ⟶ and its later distribution in Guatemala (lake Izabal area and surroundings of city of Metapa), broken line with arrow ----->. From Guatemala the disease advanced to Mexico to the state of Chiapas, first reported close to the city of Tapachula, later in Villa Flores in 1987 and finally close to the city of Teapa, State of Tabasco in 1991. Areas where Moko disease exists are marked with circles.

very probable introduction is by man that had taken contaminated material to the region.

Control measures were immediately employed in Tabasco, and the diseased plants were eliminated, so that this disease would not extend to other banana region.

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Epidemiology and control of blast of dormant flower buds of pear incited by *Pseudomonas syringae* pv. *syringae*

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ABSTRACT

Dormant flower bud necrosis of commercial pear orchards is a disease that results in significant economic losses in certain fruit tree growing areas, and specially in Catalunya, Spain. The disease has both a pathological and a host-related component that acts depending on environmental-agronomic conditions. The pathological component has been mainly attributed to infections by *Pseudomonas syringae* pv. *syringae.* The host related component has been associated to disequilibria during flower bud induction and differentiation periods and specially to abnormalities in the contents of boron, calcium, potassium and water soluble sugars in flower buds during winter dormancy.

Infectivity tiration experiments performed upon microinjection of dormant buds from cut branches forced to bloom, indicated that the strains were capable of developing the symptoms of disease observed in the field. However cold temperature shock and inoculum dose had a significant effect on the final disease incidence. Winter populations of *P. syringae* in dormant flower buds ranged from less than 10^3 to 10^8 cfu/g f.w. Ice nuclei active at - 5 C were associated with populations of *P. syringae* during 1988, 1989 and 1990 and ranged from undetectable to about 10^5 i.n./g f.w. Mean nucleation frequency was 3.2 x 10^3 cfu/ice nucleus. A significant relationship was found for six orchards and three cultivar types during the period of 1988-1989 among the population levels of *P. syringae*,

the amount of ice nuclei in dormant flower buds at the start of winter time, and subsequent disease incidence measured as bud failure assessed in early march after several winter frosts. However this relationship was not significant for the period 1989-1990 suggesting that the host dependent component would be important at this particular period.

Attemps to control disease by means of bactericidal sprays has been performed since 1985. Standard copper or antibiotic bactericidal sprays applied during full, winter or spring (prebloom stages) has not been succesfull. However, recently aluminium tris-O-ethyl phosphonate, a compound that has been reported to cause a stimulation of host defense responses in some plants, shows an average efficiency of 50% in reducing the incidence of dormant bud failure. This result reinforces the hypothesis that the disease is highly dependent on a pathologic and on a host-dependent component.

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Meteorological factors influencing bacterial blight of cowpea

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ABSTRACT

Influence of meteorological factors on bacterial blight (*Xanthomonas campestris* pv. *vignicola*) of cowpea (*Vigna unguiculata*) was studied during years 1986 and 1987. Disease appears in *Kharif* (rainy) season only. For the prediction of disease, a model $Y = 35.106 + 0.038\ X_1 + 0.094\ X_2 + 0.083\ X_3 + 0.098\ X_4$ was developed by linear regression analysis where Y, X_1, X_2, X_3 and X_4 were disease intensity, minimum temperature, maximum temperature, minimum RH and maximum RH, respectively. All the explanatory variables influenced the disease developent significantly and explained 37.6% of variation.

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Common blight of bean
Experiments and results on *Xanthomonas campestris* pv. *phaseoli*

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ABSTRACT

The common blight of bean (*Xanthomonas campestris* pv. *phaseoli*) is an important disease in Swiss horticulture. Some years ago the pathogen was introduced by contaminated seed material.

Field and laboratory experiments were carried out for three years with the aim to find the best isolation methods of the pathogen and show the susceptibility of the different and most common bean varieties.

There was not one variety of beans which was absolutely resistant against the disease.

In older stages of the disease the different bean varieties did not show clear differences in the intensity of the symptoms.

The disease symptoms developed slowlier on the fruit pods than on the leaves.

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Distribution of races of pea bacterial blight (*Pseudomonas syringae* pv. *pisi*) among protein peas in France

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The pea bacterial blight has been known to affect protein peas in Western Europe since 1985. As France was formerly considered free from the disease, the occurrence of races was unknown. Recently, the distribution of races has been investigated with respect to the broad range of winter and spring cultivars grown in the country. So far, races 2, 4 and 6 of the seven races already described, were found. Race 2 predominates in the Northern half of France where spring cultivars are grown whereas race 6 is prevalent in the South–West of the country where mostly winter cultivars are grown. Race 4 was identified in both areas but in smaller amounts than the other races.

This irregular distribution is attributed to the susceptibility characteristics of winter and spring cultivars. These two types of cultivars differ from each other in their susceptibility patterns although, among a given type, cultivars show a noticeable similarity due to their common parental background.

Resistance to race 6, with the wide host range, is lacking in the spring and winter cultivars grown at present or likely to be grown in a near future.

Although not yet widely utilized, resistance to several races, namely the dominant race 2, is commonly available in the pea germplasm. The release of new cultivars with different susceptibility patterns or the introduction of other races with seed importation, could modify the present race distribution.

Search for resistance sources and monitoring the natural populations of the pathogen constitute a strategy which is now under investigation in some EEC countries for the future control of the disease and the prevention of spread and outbreak of races.

VI

PGPR - Biological, chemical and integrated control

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Chemical and biological control of bacterial diseases: a need?

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ABSTRACT

The control of bacterial diseases is currently achieved through the use of prophylactic and cultural practices, associated when necessary with the choice of resistant varieties. Chemical control (and biological control, when available) is often considered to be subsidiary, for a number of reasons.

Among these reasons is the limited effectiveness of these methods. This is linked to the very nature of bacterial diseases of plants : relatively low economic importance, often erratic in their level of severity and difficult to predict. The nature of chemical, as well as biological, agents used against bacteria is also a source of specific difficulties.

Although numerous efforts have been made in recent years to tackle these difficulties, (new active ingredients, new antagonistic strains, improved warning systems, better detection of the pathogens), it is obvious that it will be difficult, if not impossible, to render this kind of control as simple and practical as it is in the case of most fungal diseases.

The advances already made in infection mechanisms, basis of pathogenesis and plant response to aggression, concerning a number of bacterial diseases give us hope. A control, either chemical or biological, could now be based on a logical, instead of empirical approach : a chemical could be selected for its role in a precise step of the plant-bacteria interaction. Similarly, a biological control agent could be especially created by genetic engineering for the specific control of a disease ... etc.

These are undoubtedly very fascinating and promising lines to follow up. They could lead to new solutions but it remains possible that genetic control (creation of resistant cultivars) will benefit more from these new data than chemical and biological control, at least for certain diseases.

Finally, we must remind ourselves that a preliminary to any significant improvement in the control of bacterial diseases is that the need of such control is clearly expressed by the economic operators involved.

KEY-WORDS : Bacterial diseases of plants, chemical and biological control

In this contribution, I do not intend to present a review of what is available in the field of chemical and biological control of bacterial diseases. I would rather like to summarize briefly the general features of these types of control, and to try to examine to what extent they reach their aim, how far they could be improved (and for what reason they need to be improved) and finally on which principles a more effective control of these diseases could be based.

Obviously the economic importance of a disease is linked to the degree of sophistication -and therefore the cost- of the control measure which can be used to reduce its damages. This is why it is important to keep in mind the economic aspects when considering the scientific or technical possibilities of control.

In order to limit the scope of this paper, I have chosen to exclude a number of control techniques which I do not consider here as 'real' biological or chemical techniques, for example, the biocontrol achieved by rhizobacteria (PGPR), which is presented in another contribution in this session, and whose use -although considered as biological control- is specific to the group of soil pathogens (mainly fungi). Similarly, the chemical treatments for disinfection of soil, seeds, or tools are not accounted for here. In addition, control of intracellular microorganisms (MLO, BLO) whose biology is very different from the biology of extra cellular pathogenic bacteria, will not be examined.

My comments on chemical control will be mainly illustrated by examples chosen in the control of fire blight (*Erwinia amylovora*) of apple and pear. *Agrobacterium tumefaciens* will provide the example of biological control.

1. PRESENT STATUS OF CHEMICAL AND BIOLOGICAL CONTROL

A. CHEMICAL CONTROL

• *General principles*

The aim of chemical control is to kill the bacteria before they succeed in penetrating their host, either by the destruction of epiphytic bacterial population, by preventing the production of inoculum from active lesions (*i.e.* : ooze, exudate ...) with a spray applied on existing lesions (cankers, necrosis), or by protection of potential invasion sites (stomates on leaves, nectaries in flowers, wounds ...), with a preventive application on healthy plants, before their contamination by the bacteria.

Systemic chemicals having the ability to act against the bacteria inside the plant are not currently available, although some chemicals may penetrate the plant quite easily (phosetyl-Al) or at least partially (streptomycin).

• *Active ingredients*

When reviewing the literature on chemical control of bacteria, one is first impressed by the number of different active ingredients which are always being proposed for experimentation. For example, GARRETT (1990) produced a table of active ingredients or commercial products which have been tested against fire blight during the last 10 years , but later shown to be ineffective. This list includes nearly 40 names of chemicals each of which has been said to be active against the disease, but recognized as ineffective after experiment.

If we continue with the example of fire blight, we could similarly propose a list of chemicals which are really effective against the disease, but which never reached the phytosanitary market, for 'certain' reasons. This list would include a number of antibiotics, plant extracts, other chemical compounds, organic acids, fungicides, heavy metal, salts etc ...

Therefore if we limit our list of chemicals to those registered and actually in use commercially and routinely, at least in few countries, against bacterial diseases, we will have quite a limited number of chemicals which can be

classified into 3 groups : i) heavy metals : Cu (a number of cupric salts and compounds), ii) antibiotics : streptomycin, flumequine, kasugamycin ..., iii) other chemicals : phosetyl-Al, ...

It is very likely that a few more compounds used in certain places against certain diseases could be added to this list. But these amount to few (if candidate chemicals are excluded) to complete this table.

• ***Method of application***

All these chemicals are preventive. They must be applied on the host plant before the infection or soon after. They are all of significant effectiveness, and some of them have long been used against bacterial diseases, (streptomycin : 1950, copper, 18th century). An example of the results obtained against fire blight with some of these is given in table 1 which illustrates typical results of experiments with chemicals against bacterial diseases.

Table 1 - Chemical control of blossom blight on pear and apple
Results obtained after artificial inoculation (1988-1990)

Chemical dose	Control (inoculated and unsprayed)	Bordeaux 1000 ppm	Plantomycin 100 pm	Firestop 300 ppm	Aliette 4000 ppm
Experiments	% infected blossoms/untreated control				
1	100a	61b	30c	55b	35c
2	100a	78ab	60b	NT	45c
3	100a	51b	37c	NT	33c
4	100a	63b	26c	33c	23c

NT : not tested (from PAULIN *et al*, 1990)

Observations based on this data may be applied to other diseases

1. Experimental procedure. Like most of those given in the literature, these results are obtained only after artificial inoculation of the bacteria, combined with application of the chemical : such experiments can only give a relative assessment of the value of the chemicals under experimental conditions. Results in field activity concerning chemicals are scarce, whatever the disease concerned.

2. Level of effectiveness. 100 % effectiveness is never attained at a dosage which is compatible with cost, hazard and phytotoxicity problems. Variations are observed between experiments, even with the most active chemical, and despite the controlled experimental conditions.

3. Active ingredients. The oldest chemical (copper), being still in use, is still under experiment : this is another indication that the level of control achieved by these chemicals is not satisfactory.

4. Mode of action of these chemicals. With the exception of Phosetyl-Al (Aliette), these chemicals act on the bacteria itself. It has been suggested that copper compounds may also induce a certain level of resistance to the infection in the plant tissue. The mode of action of Phosetyl-Al, in the case of bacteria, is unknown. It has a high Minimal Inhibitory Concentration (MIC) *in vitro* for bacteria. For fungal diseases it is supposed to induce defence mechanisms in the plant, either directly, or by increasing the production of elicitors by the pathogen. In addition, it may have a certain level of antibiotic activity *in planta.*

Chemicals presently in use are therefore far from perfect. Even when applied at the right moment on the plant, after artificial inoculations in experiments, they rarely allow total control, and none of them is entirely reliable.

• Timing of sprays

Consequently, the timing of sprays is a key factor in the quality of the control. Several types of schedules can be recommended, according to the type of disease to be controlled, the local technical support, and the value of the crop to be protected :

i) Systematic schedules (calendar based, or phenology based) which imply a number of applications of the chemical at dates determined irrespective of the local development of the disease (sprays applied for safety), and ii) Disease based schedules, which rely on the epidemiology of the disease, may work either 'manually', or automatically, with the help of a

computer. The aim of such schedules is : to reduce the number of sprays to only those which are needed ; to increase the effectiveness of each spray by an appropriate timing of the applications aimed at reaching the bacteria when they are most vulnerable to the action of the chemical (*i. e.* before penetrating the plant).

Factors playing a role in the epidemiology of the disease are taken into account in these systems. They belong to 3 categories : climatic factors (potential of the disease, infection periods), plant factors (susceptibility of the species or cultivars, phenological stage, receptivity to the disease according to the stage of growth), pathogen factors (virulence of the strains, expected amount of inoculum ...).

Ideally, the results of these systems should be a climatic analysis of the potential of the disease, an indication of the level of risk, and advice for control : to spray or not, when, which chemical, or other control measures.

Such systems already exist for some bacterial diseases, although they are much more common for fungal diseases. They are generally known as models, because they try to adjust certain phases of the life of the pathogen to mathematical functions. For fire blight of pear and apple, such systems have recently become available in the United States (STEINER, 1990). A computerized system is being tested in France on a different basis and includes weather forecast, (JACQUART & PAULIN, 1991). Several other warning systems for fire blight are proposed in Europe (PAULIN & BILLING, 1990).

• ***Specific problems associated with chemical control***

- Level of control

The level is usually considered too low. This can be due to several causes : lack of sufficient level of toxicity of the chemical for the bacteria (*in vitro*) ; wrong localization of the chemical on or in the plant (far from the infection site), wrong timing of the spray (too early or too late with respect to infection).

- The resistance problem

This problem is well known with antibiotics (chromosomal, plasmidic), and induces the limitation of their use in certain countries (transferable resistances). Resistance to copper salts has been recently pointed out in several bacteria (*Pseudomonas, Xanthomonas*), and this can be of great concern in the countries where these chemicals are routinely used.

- The environmental question

This question arises with any type of phytosanitary use of chemicals. It encompasses the problems of residues, of transferable resistance and of human toxicity during the application in the field.

• ***An ideal bactericide***

In order to stimulate phytosanitary firms in their research for compounds against bacterial diseases, GARRETT (1990) produced a list of characteristics which a bactericide should possess to tackle these difficulties (table 2).

Table 2 - Main traits of an improved compound against bacterial diseases

"Ideal" bactericide (from Garrett, 1990)	
1.	Cheap
2.	Wide host range
3.	Some systemic action (at least)
4.	Reaches infection sites
5.	Does not induce resistance of the pathogen
6.	Does not allow selection of plasmids with resistance genes
7.	Not of medical or veterinary use
8.	Complies with national and international regulations for registration

B. BIOLOGICAL CONTROL

It may be interesting to highlight first the specific situation of biological control, in the case of bacterial diseases : while for insect pests, and for fungal diseases, biological control has been promoted or attempted as an answer to

environmental problems (the main aim was to reduce the pollution or health hazard), in the case of bacterial diseases, biological control has usually been attempted because classic (chemical) techniques did not provide a satisfactory level of control.

• *Examples of biological control*

As is the case for chemical compounds, experiments in biological control are many, and very diverse. They are aimed at the control of numerous diseases with different biological agents. But the number of biological controls actually used is even more restricted than for chemical controls. In fact only one is widely available (*Agrobacterium tumefaciens/A. radiobacter* K84), as well as one said to be used in Australia (bacterial blotch/*P. fluorescens*) (table 3).

Table 3 - Biological control of bacterial diseases

Commercially available
• *A. tumefaciens/A. radiobacter* (K84, K1026)
• *Pseudomonas tolaasii/P. fluorescens*
Proposed for experiments
• *P. syringae* pv. *syringae* (INA+)/deleted *P. syringae* pv. *syringae*
• *P. solanacearum*/avirulent mutants
• *E. amylovora/E. herbicola*
• *X. campestris* pv. *pruni*/bacteriophages
• *X. campestris* pv. *oryzae*/antagonistic bacteria

• *Mode of action of biological control*

The activity of a biological agent against a bacterial disease may be expected from two main modes of action, which can act in conjunction with each other : i) an action upon the pathogen itself is obtained by the use of non specific bacterial antagonists (*Bacillus* sp., fluorescent Pseudomonads, *Erwinia herbicola* are the most frequently cited bacteria). Other microorganisms may play a role (fungi, moulds). In some cases, specific antagonists are used (bacteriocin producers, such as *A. radiobacter* K84).

Infections of the pathogens by parasites have been attempted in several cases ; the most widely experimented bacterial parasites are bacteriophages, although some experiments with the parasitic bacterium *Bdellovibrio* have been tried. ii) On the other hand, an action on the plant, or during the infection process, can result from the application of a biological agent : this is the case when we use avirulent variants of the pathogen (*P. solanacearum*, *E. amylovora*, *A. radiobacter*), genetically engineered pathogens (*P. s.* pv. *syringae*), or incompatible pathogens.

• *Is the case of A. tumefaciens/A. radiobacter unique ?*

The biological control of crown gall with the strain K84 of *A. radiobacter* is very well documented. Its main features are : it has been in use since 1973, and was first applied in Australia. It is considered to be at least as effective as chemical control, but less expensive. The control is preventive, and results from a simple dipping of plants in suitable bacterial suspensions, in the nurseries. However *A. radiobacter* K84 does not control every crown gall, but only the one which is caused by strains of biovar 2, which are agrocinopine-nopaline strains. These are the most frequent strains in nurseries and orchards. A potential cause of breakdown of the control (transfer of the plasmid pAg K84 which carries the gene of agrocin production to pathogenic *A. tumefaciens* strains) is tackled by the use of K1026, a genetically engineered strain of K84, lacking genes for transfer of the pAg K84 plasmid.

• *Specific problems associated with biological control*

The problems which may arise with biological control could be better shown *a contrario* by the examination of the conditions of the development of the *A. tumefaciens* control. The factors which contribute to this development -even if we set aside the unusual capacities of the strain K84, and the talent and sagacity of its inventors- are multiple (table 4).

Table 4 - Some factors which contribute to the development of biological control of crown-gall

1.	Widespread disease of economic importance for a number of plants
2.	Soil disease ('stable' inoculum)
3.	Control applied in the nursery, with a single and simple operation
4.	*A. radiobacter* easy to handle
5.	Limitation of its uses to certain strains is clearly understood
6.	Breakdowns of control are explained (and therefore properly managed)
7.	Considerable amount of knowledge on *A. tumefaciens* and its infection process, strains, pathogenicity, etc ... is available

In fact the difficulties which might be tackled by a biological agent, before its use is widely accepted, are numerous. They are linked to the biological agent itself : its massive production, its conservation, its stability ; the difficulties of practical use of living organisms (registration, potential environmental hazard, cost) ; and eventually to the problems of the use of genetically engineered microorganisms. They can be as well associated with the control : possible lack of regularity, due to the complexity of the system (pathogen/biological agent/plant), and the mode of application on the plants. In addition, the biological control is often specific for a given disease, and therefore the market is usually restricted. To these conditions we can add that the phytosanitary industry does not seem very interested. But, due to the increasing importance of bio-engineering, this problem could be in the process of being solved.

C. CONCLUSION

This brief description of the present status of chemical and biological control against bacterial diseases of plants, although incomplete, shows the limitations of these techniques, associated with the active agent (chemical or biological), and with the problems of the timing of the application, which is partially solved in the case of few diseases.

The unreliability of these kinds of control is reflected in the commonplace statement that the control against bacterial diseases in practical agriculture, with very few exceptions, does not rely on chemical or biological techniques. The control is normally achieved by the application of prophylatic rules, or cultural practices, and by the use of resistant species or cultivars.

This does not mean, however, that chemical and biological control must be neglected.

2. POSSIBLE IMPROVEMENTS

GENERAL CONDITIONS FOR IMPROVEMENTS

• *Economic conditions*

The chemicals used (or experimented with) against bacterial diseases have in common the fact that none of them was initially developed to be a bactericide for plant pathology. They are all by-products of another field of activity, often of medical or veterinary use (streptomycin, flumequine), sometimes from another section of plant pathology (copper salts, phosetyl-Al). This underlines the fact, often pointed out, that there is not a sufficient market for the phytosanitary industry in bacterial disease of plants (as compared to herbicides, insecticides or fungicides). It is sometimes considered that even a compound which would work against a wide range of diseases (*i.e.* streptomycin) would not be an attractive enough proposition for chemical firms.

Efforts have been made to translate into figures the losses induced by bacterial diseases of plants (KENNEDY & ALCORN, 1980). However, it would be necessary to go further than this and estimate the reduction of loss which can be expected from the use of chemical and biological control : it is this figure which would in fact represent the need for chemical or biological control. It is possible that, if the controls obtained were 100 % reliable, it would be easier to convince economic operators of the benefit of their use in this field. But, at present, no chemical or biological control is 100 % reliable !

• *Technical and scientific conditions*

The techniques described in this paper are all based on the knowledge of the epidemiology of the disease to be controlled : they try to stop the disease cycle when the bacteria has not yet penetrated into its host. This, in spite of the use of warning systems, makes the control difficult, because the presence of the target (the bacteria) is not certain, but supposed.

In recent years, most effort has been spent on explaining the mechanisms involved in the plant-bacteria interaction. Genetics and molecular-biology of infections are in the process of being discovered for several bacterial diseases. Therefore a basis now exists to establish a specific control of bacterial diseases.

POSSIBLE BASIS FOR A SPECIFIC CONTROL OF BACTERIAL DISEASES

A number of possibilities that could be followed up in order to attempt such a specific control are summarized on table 5. A control could be obtained by interventions at different stages of the infection process, and even when the bacteria has entered the plant.

Table 5 - Possible bases for a specific control of bacterial diseases

- **Before infection**
  - Epiphytic stage (non-induction of genes involved in epiphytic multiplication)
  - Infection site (prevention of chemiotactism)
- **During the infection process**
  - Non-induction of pathogenicity genes (*hrp*)
  - Modification of receptors
  - Induction of defense reactions (elicitation)
- **After infection**
  - Increase of defense reaction effectiveness in plants
  - Prevention of the multiplication of virulent bacteria in the plant
  - Inhibition of the toxic effect of a toxin

A control acting on the interaction between bacteria and plant can be expected to be more specific, and therefore more effective. Some problems remain to be solved before we can judge whether this represents a new hope for control or not. Among them, the screening for active molecules can be expected to be far more difficult than the screening for classical molecules (bactericides).

IS CHEMICAL AND BIOLOGICAL CONTROL THE BEST APPROACH TO SPECIFIC CONTROL ?

We are not sure that the achievement of specific control based on the plant bacteria interactions will be very feasible with chemical or biological control. Once such a sophisticated knowledge of the causes of the disease and the reasons for the susceptibility of the plant has been acquired, it is possible that the best way to apply this knowledge to control would be to modify the host plant itself, by breeding, or by genetic engineering. This would produce a stable, safe, and reliable control. What is more, the economic operators (seed industries, nurseries, breeders) seem to be prone to adding original improvements to their new creations : resistance to a bacterial disease can be an attractive additional feature for a new cultivar.

CONCLUSION

It seems, therefore, that chemical and biological control of plant bacterial diseases is condemned -at least in the near future- to remain limited to the use (which can be optimized) of few active ingredients, non-specifically designed for this purpose, and to some 'large-scale' experiments of biological control, for a couple of diseases.

In spite of the very significant advances already obtained, or easy to forecast, in the explanation of mechanisms, in the localization of genes and in the isolation of molecules involved in the process of pathogenesis, it is unlikely that chemical or biological control of bacterial diseases will benefit drastically from these new data. Actually, it is not sure that chemical and biological control would be the best techniques to integrate them in the resolution of the bacteria-plant interaction. Genetic control could possibly be a better proposition in making the best of these advances.

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Biological control of *Xanthomonas oryzae* pv. *oryzae*

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Abstract

The evaluation of nine microorganisms namely, *Bacillus subtilis, Erwinia herbicola, Enterobacter aerogenes, Micrococcus* sp., *Pseudomonas fluorescens, Aspergillus flavus, Cladosporium cladosporioides, Penicillium oxalicum* and *Trichoderma harzianum* along with Streptocycline (100 µg ml^{-1}) for the control of *X. oryzae* pv. *oryzae* under field conditions during *Kharif* 1989 and 1990 revealed that all the antagonists significantly reduced the disease intensity with a corresponding increase in grain yield during both the years. On an average, *B. subtilis* proved most effective in controlling the disease in both the years and provided 18.91 and 21.64% disease control alongwith 5.32 and 6.39% increase in grain yield in *Kharif* 1989 and 1990, respectively. Among all the treatments, the application of *B. subtilis* 24 hr before pathogen inoculation was found most effective in both the years and gave 35.47 and 35.51% disease control and 9.36 and 11.74% increase in grain yield in *Kharif* 1989 and 1990, respectively. On the basis of mean values of the antagonists for time intervals, 24 hr pre-inoculation application proved most promising followed by simultaneous application, post-inoculation application being least effective. All the antagonists, except *A. flavus, Micrococcus* sp., *C. cladosporioides* and *E. aerogenes*, during *Kharif* 1989 and all, except *E. aerogenes, Micrococcus* sp. and *C. cladosporioides*, during *Kharif* 1990 proved superior to Streptocycline in controlling the disease.

Key Words: Biological control, *X. oryzae* pv. *oryzae,* Rice, Bacterial blight

Introduction

Bacterial blight of rice caused by *Xanthomonas oryzae* pv. *oryzae* is the most serious disease of rice culture and causes exorbitant yield losses in tropics (WAKIMOTO, 1975). Inspite of testing of a large number of protective and systemic chemicals including antibiotics for the control of the disease, very little success has been achieved in this regard. The control of the disease through the development of resistant varieties is equally disappointing (OU, 1985).

Keeping in view the lack of effective chemical control measures and non-availability of rice cultivars resistant to *X.oryzae* pv. *oryzae,* the present investigation was planned to manage the disease through the biological control and the results obtained are presented below.

Material and Methods

Out of thirty-two microorganisms comprising 20 fungal and 12 bacterial species, isolated from phylloplane and spermosphere of 4 rice cultivars and from phylloplane of four weeds and tested *in vitro* for their antagonistic activity against *X. oryzae* pv. *oryzae*, nine microorganisms found antagonistic to the test bacterium were evaluated for its control under field conditions. Streptocycline (100 $\mu g\ ml^{-1}$) was also included as a standard check.

Rice cv. PR 106 was grown in *Kharif* seasons of 1989 and 1990. Thirty-day-old seedlings, @ two seedlings per hill, were transplanted with 15 x 20 cm spacing in 3 x 2 m^2 plots. The bacterial antagonists and *X. oryzae* pv. *oryzae* were grown in potato sucrose peptone broth for 48 hr in shake cultures at 27 $\pm$ 2 ^{o}C. The cells of bacterial antagonists and of *X. oryzae* pv. *oryzae* were collected by centrifugation and resuspended in sterilized distilled water to obtain 6×10^7 and 6×10^6 CFU ml^{-1}, respectively. The fungal antagonists were grown in potato dextrose broth for 7 days in still cultures at 25 $\pm$ 2 ^{o}C and filtered. After filtering, the fungal mat was blended in sterilized distilled water and adjusted to contain 6×10^7 CFU ml^{-1}. The antagonists were sprayed on the plants 0, 24 hr before and after the pathogen was clip inoculated (KAUFFMAN *et al.*, 1973) at maximum tillering stage of the crop.

The disease intensity on the top three leaves of 33 randomly selected plants of each plot was recorded 30 days after pathogen inoculation, using a disease rating scale of 0-9. The disease intensity was calculated using the following conversion formula:

$$\text{Disease intensity} = \frac{\text{Sum of all the numerical ratings}}{\text{Number of leaves examined} \times \text{Maximum rating}} \times 100$$

The grain yield of each treatment was recorded at the time of harvest in tonnes hectare $^{-1}$ at 14% moisture level. The data was analysed using split plot design.

Results:

The results obtained during *Kharif* 1989 revealed that all the antagonists significantly reduced the disease intensity with a corresponding increase in grain yield (Table 1). On an average, *B. subtilis* proved the most effective antagonist and brought down the disease intensity to 32.72% from 40.35 obtained in untreated check plots, alongwith 5.32% yield gain*. *P. oxalicum* with a disease intensity of 33.94% was the second best antagonist, whereas *Micrococcus* sp., *C. cladosporioides* and *E. aerogenes* with disease intensities of 39.11, 38.64 and 38.62%, respectively, were least

effective. On an overall basis, *B. subtilis*, *P. oxalicum*, *E. herbicola*, *T. harzianum* and *P. fluorescens* proved superior to Streptocycline in controlling the disease. On the basis of mean values of antagonists for time intervals, 24 hr pre-inoculation application proved most promising followed by simultaneous application, post-inoculation application being the least effective.

A perusal of the data (Table 1) reveals that the application of *B. subtilis* 24 hr before pathogen inoculation was most effective and provided a disease control* of 35.47% with 9.36% increase in grain yield. However, its simultaneous application with the pathogen resulted in only 14.34% disease control and 4.31% yield gain, post-inoculation application being the least effective. *P. fluorescens*, when applied 24 hr prior to pathogen inoculation, proved second best providing 30.63% disease control and 8.16% increase in grain yield. The pre-inoculation application of *E. herbicola* ranked third in its efficacy against the pathogen, and provided 27.78% disease control and 6.66% yield gain.

Only Streptocycline and three antagonists, viz. *P. oxalicum, T. harzianum* and *C. cladosporioides*, when applied simultaneously with the pathogen, proved superior to their pre- and post-inoculation applications. The simultaneous application of *P. oxalicum* gave the highest disease control of 23.74% and yield gain of 9.74%, followed by that of *T. harzianum* for which the corresponding figures were 20.46 and 6.08%, respectively.

The results of the trial conducted during *Kharif* 1990 (Table 2) reveal that again all the treatments significantly decreased the disease intensity resulting in a corresponding increase in grain yield. On the basis of mean values, *B. subtilis* was the most promising antagonist and reduced the disease intensity to 27.88% from 35.58% obtained in check alongwith a yield gain of 6.39%. *E. herbicola* and *P. oxalicum* were found to be the next best antagonists lowering the disease intensity to 29.13 and 29.17% and causing the yield gain of 4.44 and 4.32%, respectively; *E. aerogenes* and *C. cladosporioides* were the least effective. All the antagonists, except *E. aerogenes*, *Micrococcus* sp. and *C. cladosporioides*, proved superior to Streptocycline in controlling the disease. On an average, pre-inoculation application of the antagonists proved most effective and the post-inoculation application the least.

The maximum disease control of 35.51% and yield increase of 11.74% were obtained with pre-inoculation application of *B. subtilis*. Pre-inoculation application of *P. fluorescens*, yielding a disease control of 31.76% and yield increase of 8.57%, proved second best followed by *E. herbicola* which gave 29.00% disease control and 7.50% increase in yield. The simultaneous applications of three antagonists, namely *P.*

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* Disease control and yield gain derived as per cent of the difference between those recorded in treatment and check plots

Table 1. Field evaluation of antagonists and Streptocycline for control of bacterial blight of rice and their effect on grain yield during *Kharif* 1989

Treatment	Disease intensity (%)*				Grain yield (t ha^{-1})**			
	Antagonist's application (hr before/after pathogen inoculation)				Antagonist's application (hr before/after pathogen inoculation)			
	24 hr before	0 hr	24 hr after	Mean	24 hr before	0 hr	24 hr after	Mean
Bacillus subtilis	22.67	35.83	39.67	32.72	6.12	5.78	5.61	5.84
	(28.43)	(36.77)	(39.04)	(34.74)	(9.36)	(4.31)	(2.28)	(5.32)
Enterobacter	31.03	41.10	43.73	38.62	5.81	5.54	5.50	5.62
aerogenes	(33.85)	(39.87)	(41.10)	(38.37)	(3.66)	(-0.05)	(0.20)	(1.27)
Erwinia herbicola	25.37	36.37	42.47	34.74	5.97	5.72	5.55	5.75
	(30.24)	(37.09)	(40.67)	(36.00)	(6.66)	(3.17)	(1.06)	(3.63)
Micrococcus sp.	32.43	41.10	43.80	39.11	5.69	5.59	5.53	5.60
	(34.72)	(39.87)	(41.44)	(38.68)	(1.57)	(0.79)	(0.71)	(1.02)
Pseudomonas	24.37	40.20	43.20	35.92	6.06	5.60	5.54	5.73
fluorescens	(29.58)	(39.35)	(41.09)	(36.67)	(8.16)	(1.06)	(0.95)	(3.39)
Aspergillus flavus	29.57	37.30	43.87	36.91	5.81	5.75	5.50	5.69
	(32.94)	(37.64)	(41.48)	(37.35)	(3.79)	(3.68)	(0.20)	(2.56)
Cladosporium	33.13	39.23	43.57	38.64	5.68	5.64	5.51	5.61
cladosporioides	(35.14)	(38.78)	(41.30)	(38.41)	(1.38)	(1.80)	(0.38)	(1.19)
Penicillium oxalicum	28.13	31.90	41.80	33.94	5.83	6.08	5.57	5.83
	(32.03)	(34.40)	(40.28)	(35.57)	(4.02)	(9.74)	(1.46)	(5.07)
Trichoderma	29.20	33.27	42.33	34.93	5.76	5.89	5.56	5.73
harzianum	(32.71)	(35.20)	(40.59)	(36.58)	(2.77)	(6.08)	(1.35)	(3.40)
Streptocycline	33.03	36.07	42.13	37.08	5.69	5.74	5.57	5.67
100 $\mu g\ ml^{-1}$	(35.04)	(36.91)	(40.47)	(37.48)	(1.52)	(3.52)	(1.53)	(2.19)
Check	35.13	41.83	44.79	40.35	5.60	5.52	5.49	5.54
	(36.35)	(40.30)	(41.59)	(39.41)	(-)	(-)	(-)	(-)
Mean	29.46	34.02	42.79	36.56	5.82	5.72	5.54	5.70
	(33.29)	(35.62)	(40.85)	(37.13)	(3.89)	(3.10)	(0.91)	(2.65)
CD (p 0.05)								
Treatments				(0.345)			0.067	
Time intervals				(0.175)			0.044	
Time intervals for a fixed treatment				(0.581)			0.145	
Any pair of combination				(0.579)			0.135	

* Figures in parentheses are Arc Sine $\sqrt{\text{Percentage}}$ transformed values

** Figures in parentheses are per cent increase in grain yield over check

Table 2. Field evaluation of antagonists and Streptocycline for control of bacterial blight of rice and their effect on grain yield during *Kharif* 1990

Treatment	Disease intensity (%)*				Grain yield (t ha^{-1})**			
	Antagonist's application (hr before/after pathogen inoculation)				Antagonist's application (hr before/after pathogen inoculation)			
	24 hr before	0 hr	24 hr after	Mean	24 hr before	0 hr	24 hr after	Mean
Bacillus subtilis	20.10	30.30	33.23	27.88	6.90	6.20	6.01	6.37
	(26.64)	((33.40)	(35.20)	(31.75)	(11.74)	(3.75)	(3.68)	(6.39)
Enterobacter aerogenes	28.33	35.85	38.01	34.06	6.33	5.98	5.80	6.03
	(32.16)	(36.78)	(38.07)	(35.67)	(2.46)	(0.07)	(0.07)	(0.87)
Erwinia herbicola	22.13	30.55	34.70	29.13	6.64	6.17	5.94	6.25
	(28.06)	(33.56)	(36.09)	(32.57)	(7.50)	(3.28)	(2.54)	(4.44)
Micrococcus sp.	29.87	35.73	38.23	34.61	6.24	6.02	5.83	6.03
	(33.13)	(36.71)	(38.19)	(36.01)	(1.07)	(0.70)	(0.57)	(0.78)
Pseudomonas fluorescens	21.27	35.27	37.88	31.47	6.70	6.04	5.84	6.19
	(27.46)	(36.43)	(37.98)	(33.96)	(8.57)	(1.04)	(0.74)	(3.45)
Aspergillus flavus	27.27	33.03	38.20	32.83	6.33	6.11	5.81	6.09
	(31.48)	(35.08)	(38.17)	(34.91)	(2.59)	(2.34)	(0.22)	(1.72)
Cladosporium cladosporioides	29.93	34.06	37.23	33.74	6.25	6.11	5.86	6.07
	(33.17)	(35.71)	(37.59)	(35.49)	(1.30)	(2.31)	(1.09)	(1.57)
Penicillium oxalicum	25.30	26.31	35.90	29.17	6.41	6.38	5.93	6.24
	(30.20)	(30.86)	(36.81)	(32.62)	(3.87)	(6.80)	(2.30)	(4.32)
Trichoderma harzianum	27.13	31.00	36.87	31.67	6.39	6.24	5.88	6.12
	(31.41)	(33.83)	(37.39)	(34.21)	(3.48)	(4.44)	(1.45)	(3.12)
Streptocycline 100 μg ml^{-1}	29.70	31.93	38.23	33.29	6.22	6.15	5.81	6.10
	(33.02)	(34.61)	(38.19)	(35.27)	(0.70)	(3.00)	(0.31)	(1.34)
Check	31.17	36.87	38.70	35.58	6.17	5.97	5.80	5.99
	(33.94)	(37.39)	(38.47)	(36.60)	(-)	(-)	(-)	(-)
Mean	26.56	32.81	37.02	32.13	6.42	6.12	5.87	6.13
	(30.97)	(34.94)	(37.47)	(34.46)	(3.94)	(2.53)	(1.17)	(2.55)
CD (p 0.05)								
Treatments				(0.309)			0.125	
Time intervals				(0.144)			0.062	
Time intervals for a fixed treatment				(0.476)			0.205	
Any pair of combination				(0.489)			0.206	

*Figures in parentheses are Arc Sine $\sqrt{\text{Percentage}}$ transformed values

** Figures in parentheses are per cent increase in grain yield over check

oxalicum, T. harzianum and *C. cladosporioides*, along with Streptocycline again proved superior to their pre- and post-inoculation applications.

Discussion

The success of biological control of a plant pathogen depends upon many factors namely, concentration of antagonist applied, time gap between antagonist and pathogen application and effect of environmental factors on antagonist's growth and establishment. In the present investigation, it was found that on an average basis, the application of the antagonists 24 hr before the pathogen inoculation provided significantly better protection than the simultaneous and post-inoculation applications. The better disease control by the antagonists when applied before the pathogen might have been possible due to the fact that antagonists got sufficient time for their multiplication, production of sufficient amount of antibiotics and colonization of host surface. The phenomenon of antibiosis by these antagonists was established in an *in vitro* experiment of this investigation. In addition to antibiosis, competition for nutrients and creation of acid conditions by *E. herbicola* (RIGGLE & KLOS, 1972), siderophore production and induction of host resistance by *P. fluorescens* (AHL *et al.*, 1986) and hyperparasitism by *Bacillus* spp. (LEVINE *et al.*, 1936) and *Trichoderma* spp. (DENNIS & WEBSTER, 1971) have been shown. The better disease control with pre-inoculation application of the antagonists over simultaneous or post-inoculation applications have been shown by earlier workers also (BALDWIN & GOODMAN, 1963; KLOS, 1969; MC INTYRE *et al.*, 1973).

Preliminary studies made by earlier workers to control bacterial blight of rice using biological agents have shown some promising results. NWIGWE (1973) observed reduction in pathogenicity of *X. oryzae* pv.*oryzae* by an epiphytic yellow bacterium. HSIEH & BUDDENHAGEN (1974) and SANTHI *et al.* (1987) reported suppression of symptom development by inoculating rice plants with a mixture of *X. oryzae* and *E. herbicola*. ANURATHA & GNANAMANICKAM (1987) found that coating of rice seeds and spraying the seedlings 20 days after sowing with *P. fluorescens* caused 40-60% reduction in disease severity.

The significant disease control provided by all the antagonists in the present investigation might have resulted due to better establishment of the antagonists in the niche because all the antagonists, except *Micrococcus sp.* and *T. harzianum*, were tried in their natural habitat. BLAKEMAN & FOKKEMA (1982) have also stressed that the use of naturally occurring resident microorganisms for biological control should be preferred to the organisms from other habitats which may be equally antagonistic to the pathogen.

It is also interesting to note that out of nine antagonists tried, five antagonists in *Kharif* 1989 and six in *Kharif* 1990 gave a significantly better disease control than Streptocycline, a highly potent antibiotic used widely for bacterial plant pathogens. Out

of these antagonists found superior to Streptocycline in controlling the disease, three antagonists namely, *B. subtilis, E. herbicola* and *P. oxalicum* also gave significantly higher grain yield than Streptocycline. It clearly indicates that these three antagonists have a very good potential to be tried for controlling bacterial blight on field scale.

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Xanthomonas campestris pv. *pruni* bacteriophages on peach trees and their potential use for biological control

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INTRODUCTION

Xanthomonas campestris pv. *pruni* (*Xcp*) causes bacterial spot disease of stone fruits. Summer infections of *Xcp* frequently cause leaf and fruit spot epidemics in peach orchards in Northern Italy and in particular in the Veneto region (south-east area of Lake Garda) (SACCARDI and GOIO, 1990).

Italian legislation prohibits the use of antibiotics and does not foresee the use of any of the few alternative, non-antibiotic, antibacterial compounds allowed in other parts of the world (WORTHING and HANCE, 1991). In the past, more than 20 years ago, Civerolo and Keil (1969), spraying bacteriophages on the peach trees leaves, immediately or 24h before inoculation with *Xcp*, were able to reduce disease intensity by 45%.

The object of this study was to prevent, stop or slow-down peach spot epidemics with a biological control technique based on phages.

To accomplish this, in diseased peach trees of the Veneto region, some phages associated with *Xcp* were isolated and their virulence was tested. The phage capacity to prevent fruit spotting and their survival on the leaf surface were assayed. Finally, the interference of 19 pesticides with the *Xcp*-phage interaction was tested.

MATERIALS AND METHODS

A total of 8 phages, active against *Xcp*, were isolated from samples of infected leaves collected in different orchards according to STOLP and STARR, 1964.

The sensitivity of 21 Italian and foreign *Xcp* strains isolated from peach and from

plum was assayed with 6 selected phages.

As for the protection experiments, detached nectarine fruitlets (cv. Venus) sprayed with phage F_8 at the concentration of 10^8 p.f.u./ml were air-dried for 30 min and then sprayed with a suspension of the strain VR69 of *Xcp*. Fruitlets sprayed with distilled water instead of phage suspension were used as a control. After one week the fruitlets were examined for the presence of watersoaked areas around the inoculation holes and bacteria reisolation was performed.

The partial disease symptom frequencies, that is the successful inoculations over the total ones, in untreated (control) and phage pre-treated fruitlets (treated) were calculated.

To test phage survival in the climatic chamber, 14 groups of 9 peach leaves were dipped in phage suspension and placed in Petri dishes which were kept for 1 week at 25°C. Immediately, and at 24 h intervals, 2 controls and 2 treated groups were taken and washed for 1 h at 25°C in water amended with Tween 20. After sterile filtration, the washing fluid was used for phage counting.

To test phage survival in the orchard, at the end of July 1991, a phage suspension was sprayed on the leaves of nectarine trees cv. Spring Red. As a control, an alternate row of trees was sprayed with water. At 24h intervals, for 6 days, leaf samples were taken from the control and treated trees. At each time, four sub-samples of 5g were taken from the control and treated leaves. The washing fluid from each sub-sample was used for phage counting.

As regards the interference of pesticides with the phage-bacteria interaction, this was tested *in vitro*. The 19 main chemicals used for summer treatment in peach orchards of the Veneto region were tested. The F_8 phage-VR69 strain system was used. 1ml of bacterial suspension, grown for 24h at 25°C, was placed in flasks amended with the pesticide with a final concentration equal to the maximum recommended dose. After 24h of incubation at 25°C, the phages were isolated and counted.

RESULTS AND DISCUSSION

All six phages caused clear plaques in 12 out of 17 strains of *Xcp*. Two of the remaining 5 strains were not lysed by any phage and 3 were only partially lysed.

Phage F_8 had the widest range of activity and therefore it was selected to set up a biological control technique to be adopted during the summer for the protection of leaves and fruitlets.

After 1 week the phage suspension had prevented disease symptoms in 92% of the

treated fruits. In the control fruitlets, reisolation of bacteria from the watersoaked areas was successful in 15 out of 16 cases.

On the basis of the reisolation results, partial frequencies of the presence of endophytic bacteria were obtained.

The protection induced by phage treatment might be caused either by lysis of *Xcp* cells or by lysogeny; in the latter case, lysogeny might have inhibited bacterial endophytic colonization, reducing the activity of *Xcp*. The latter phenomenon might explain bacterial reisolation from treated asymptomatic fruitlets.

In the climatic chamber, the phage population dropped progressively during the week: starting from 10^7 p.f.u./ml and reaching 10^6 p.f.u./ml after 6 days.

The survival of phage F_8 on the surface of peach leaves in the orchard was approximately 10^4 times lower than on the leaves in the climatic chamber. In the orchard, phages survived for at least 5 days. However, a further selection assured a phage survival for at least 10 days.

Out of the 19 pesticides tested, only those containing ZIRAM and TMTD completely inhibited the phage F_8. An appreciable reduction in phage population was caused by Benzoximate, Chlorpyrifos-methyl, Parathion-methyl, Triforine and Propargyl. The phage population increased with Carbendazim and Etionfencarb.

To summarize, the reisolation of phages from all 8 diseased leaf samples indicates their natural presence in the Veneto peach orchards affected by *Xcp*. The uniform response of 12 *Xcp* strains to the 6 selected phages suggests a similar phage sensitivity among the bacterial strains. The protective effect of phage spraying on peach leaves was confirmed in fruitlets but with a phage concentration 10 times lower than the one used in the past. Moreover, the same treatment gave a significant reduction in the endophytic presence of the bacteria, indicating inhibition of pulp colonization. It is likely that the phage causes a reduction in *Xcp* activity through lysogeny. Phage F8 survived on the surface of healthy peach leaves for at least 5 days. In the orchard, after 1 week, the decrease in the phage population was 10^4 times higher than that found on the leaves in controlled environment. This was probably caused by high temperature, dehydration and ultra-violet radiation. It is also possible that in the orchard some phages became passively endophytic during water congestion and therefore they were not reisolated because the technique was based only on surface washing of the leaves. The marked reduction in phage population induced by some pesticides was probably due to the reduction or inhibition of bacterial growth. However, since the pesticides tested were commercial formulations, it is impossible to say whether the positive and negative interferences were caused by the active principle or by additives.

The conclusions that can be drawn from this study indicate that phages may be

considered as candidates for *Xcp* antagonists in biological control. The selection of natural phages with a longer epiphytic survival in peach orchard and their application in appropriate quantities should offer adequate protection with weekly treatments.

In biological control, immunity given by lysogenic conversion and resistance due to the natural genic mobility of the bacterial populations, may occur when the target organism and/or the antagonist are procaryotes (VIDAVER, 1982). Therefore, when a biological control is being set up for *Xcp* in the field with the use of phages, to reduce those undesirable effects, the following measures must be adopted:

1) restrict the control to a limited peach-growing areas;
2) use selected indigenous phages and replace them frequently (probably once a year);
3) use a low number of treatments limited to the period of greatest fruit susceptibility;
4) calibrate the doses in relation to survival;
5) do not use the same phages on peaches and on plums or other stone-fruits.

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Contribution of root attachment, root colonization and production of a new agrocin by *Agrobacterium radiobacter* strains K84 and K1026 to biological control of crown gall

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Abstract

Agrobacterium radiobacter strains K84 and the Tra- deletion mutant K1026 are commercially used to control A. tumefaciens. Production of agrocin 84 by both strains is the main mechanism of biocontrol but efficiency against strains of A. tumefaciens resistant to agrocin 84 prove that other mechanisms play a role in this biocontrol. The ability of strain K84 to attach to in vitro cultured tomato root tips has been quantified and compared to the corresponding of the B6 strain of A. tumefaciens using ^{35}S-radiolabeled bacteria. A quantification of the numbers of strains K84 and K1026 has been studied in roots of stone fruit trees: roots were efficiently colonized and 10^6 c.f.u. of K84 and K1026 remained per gram of fresh weight of roots after 60 days. Production of an antibiotic-like substance, other than agrocin 84, by strains K84, K1026 and K84 Agr- (agrocin 84 non producer) has been evidenced: this substance has shown an inhibitory effect against biovar 1, 2 and 3 strains of A. tumefaciens and against other phytopathogenic and non pathogenic soil habitants.

Keywords: Crown gall, biocontrol, attachment, colonization, agrocins, Agrobacterium tumefaciens, A.radiobacter.

Introduction

The biological control of Agrobacterium tumefaciens using strains K84 and K1026 (Tra- deletion mutant of K84) of A. radiobacter was initially explained resulting from the production of an antibiotic, agrocin 84 by strain K84 (Kerr and Htay, 1974). According to the correlation sensitivity to agrocin 84-effectiveness of biocontrol, only strains of A. tumefaciens sensitive to agrocin 84, should be controlled in the field. However, different authors showed efficiency of K84 against strains of A. tumefaciens resistant in vitro to agrocin 84 (López et al, 1989; Vicedo et al, 1992). This study was undertaken to determine: (i) comparative effectiveness of strains K84 and K1026 against strains of A. tumefaciens resistant to agrocin 84; (ii) analysis of some mechanisms other than agrocin 84 sensitivity implied in the biocontrol of these strains.

Biological control

The efficiency of strains K84 and K1026 in biocontrol of two strains of A. tumefaciens resistant to agrocin 84 was studied. Rooted plants of hybrid "GF677" (peach x almond) growing under controlled conditions in open-air pots were used. Sterile substrate was artificially inoculated with isolates 678-2 or 436-2 of A. tumefaciens. The experiment was similar to those described by López et al, (1989). Fifty plants were used per treatment (control, K84 or K1026 peat preparations). Results of biocontrol are shown in table 1.

Table 1

A. tumefaciens strains	Treatment	% galled plants
436-2	Control	19.44 ± 0.06
	K84	0.00
	K1026	0.00
678-2	Control	50.00 ± 0.07
	K84	16.66 ± 0.06
	K1026	0.00

Biocontrol of crown gall was complete in plants treated with strain K1026. Full control was also observed in plants

growing in soil inoculated with the strain 436-2. In plants growing in soil inoculated with strain 678-2, some of them were galled, but differences with the control were obvious.

Root attachment

Comparative attachment of strains A. radiobacter K84 and A. tumefaciens B6 to tomato root tips in vitro cultured was studied. Root tips were incubated with a ^{35}S-methionine radiolabeled bacterial suspensions of either A. radiobacter K84 or A. tumefaciens B6. At different times radioactivity in the root tips was measured. Attachment of radiolabeled strain B6 was also studied after preincubation of root tips in different suspensions of non-radiolabeled strain K84. The number of attached bacteria was calculated according to the specific radioactivity of the bacterial suspension.

Results of the comparative attachment of K84 and B6 are shown in Fig 1. Fig 2 shows attachment of B6 with or without preincubation with K84. Attachment of K84 was significantly lower than B6. No significant differences were observed in B6 attachment with and without preincubation with K84. These results suggest that attachment of K84 is different to that of A. tumefaciens. Apparently there is not enough blockage by K84 of the infection sites as to explain biological control of strains of A. tumefaciens resistant to agrocin 84.

Root colonization

Root colonization by strains K84 and K1026 was studied in "GF677" hybrid rooted plants. Roots were inoculated with 10^9 cells/ml of K84 or K1026 and using a selective medium, the number of bacteria per gram of root was calculated for 60 days. Results are shown in Fig 3. Roots were colonized with similar efficiency by both strains and no statistically significant differences were observed. These results agree with previous experiments showing that strains K84 and K1026 are good root colonizers (Vicedo et al, 1992).

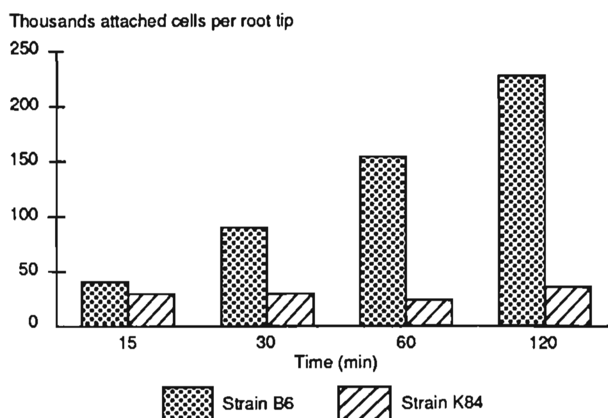

Fig.1 Attachment of B6 and K84 tomato root tips.

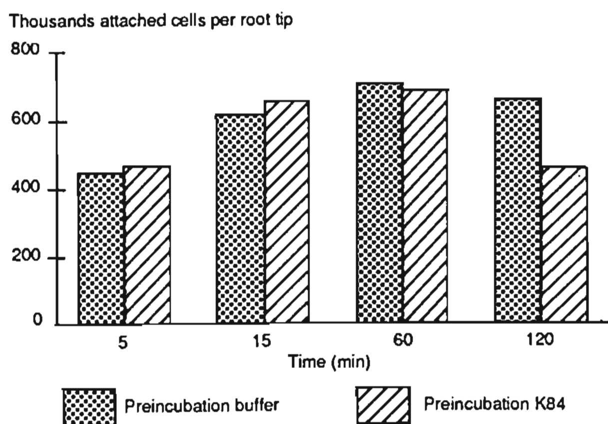

Fig.2 Attachment of B6 with or without preincubation in K84

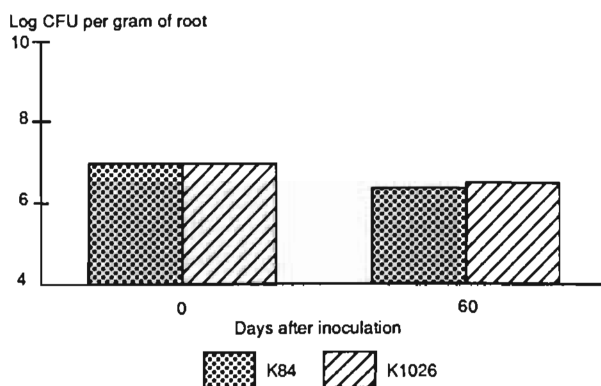

Fig.3 Colonization of "GF 677"rootstock by K84 and K1026 strains.

Production of an antibiotic-like substance other than agrocin 84.

Production of this substance was studied using the Stonier medium (Stonier, 1956) and the mannitol-glutamate medium (Moore et al, 1988). Strains K84, K1026 and K84 Agr- (cured of the pAgK84 and agrocin 84 non producer), were able to produce this substance only in mannitol-glutamate medium. This substance has a broad spectrum of activity affecting strains of A. tumefaciens biovar 1, 2 and 3 (table 2). The sensitivity seems to be not Ti-plasmid codified. No correlation between sensitivity to agrocin 84 and to the antibiotic-like substance was observed (table 2). Other plant pathogenic bacteria such as Erwinia carotovora subsp. carotovora and atroseptica, Pseudomonas corrugata, P. cichorii and Xanthomonas campestris pv. campestris (López et al, 1991) and ten unidentified saprophytic bacteria isolated from tumors were also inhibited.

Table 2

A. tumefaciens	Agrocin 84		Antibiotic-like substance	
	Sensitive	Resistant	Sensitive	Resistant
Biovar 1	11	12	15	8
Biovar 2	22	8	7	23
Biovar 3	0	3	3	0

Although biochemical composition of this substance has not been determined, its activity against different soil borne bacteria, suggests that strains K84 and K1026 could be able to create special conditions around the roots if it is produced in situ. The inhibitory effect against strains of A. tumefaciens resistant to agrocin 84, suggests an involvement in the biocontrol of these strains.

Conclusions

Strains K84 and K1026 have similar ability to control strains of A. tumefaciens resistant to agrocin 84. To explain their effectiveness, efficiency in root colonization by both strains has been observed, but not blockage of the infection sites. Strains K84 and K1026 are also producers of an

antibiotic-like substance different to agrocin 84. Biological control of crown gall is a polygenic phenomenon and strains K84 and K1026 possese many properties, in addition to the production of agrocin 84, that can contribute depending upon the circumstances, to the efficiency of the control to varying degrees.

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Advances in biological control of bacterial wilt caused by *Pseudomonas solanacearum*

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Abstract: Bacterial wilt is caused by the soilborne phytopathogen *Pseudomonas solanacearum*. Although some success in controlling the disease has been achieved by means of cultivars which are tolerant to the bacterium, the situation is complicated because expression of resistance varies according to geographic location. Consequently, during the last decade, there has been increased interest in exploring alternative strategies for crop protection.

Numerous microorganisms that can inhibit the growth of *P. solanacearum* have been described, but none of these are able to effectively control bacterial wilt under field conditions. For control of bacterial wilt the biocontrol agent must be able to establish itself under conditions that favor *P. solanacearum*. For example it must be able to colonize the root system of the host plant, to compete effectively with the naturally-occuring rhizosphere microorganisms, and to survive adverse conditions that can occur in the soil environment.

To satisfy these requirements, we have tested a series of genetically engineered avirulent mutants of *P. solanacearum*. Certain of these mutants, with lesions in the *hrp* gene cluster, retain the ability to colonize the host plant upon root inoculation and can multiply both in the rhizosphere and on the rhizoplane, as well as *in planta* (collar and lower part of the stem).

The characteristics and biological properties of these genetically engineered mutants are described in these proceedings (FREY et al.).

Keywords: *Pseudomonas solanacearum*, biological control, genetically engineered avirulent mutants.

Introduction

Bacterial wilt caused by *Pseudomonas solanacearum* E. F. Smith is a soil-borne plant disease of substantial economic importance in tropical and subtropical regions of the world. *In planta* the bacterium is primarily limited to vascular tissue and wilt occurs due to the occlusion of xylem vessels by bacterial slime and plant tyloses. Some success in controlling the disease has been achieved by using cultivars which are tolerant to the bacterium. However, the level of resistance depends on the aggressiveness of bacterium, a trait which can vary according to geographic location. Therefore, there has recently been increased interest in developing alternative strategies for control of bacterial wilt, such as the use of microbial antagonists.

Biological control mechanisms may be direct (competition and/or antibiosis) or indirect (induced resistance of the host plant). Antagonistic bacteria [*Pseudomonas fluorescens* spp., *Bacillus* spp. (CIAMPI-PANNO et al., 1989; ANURATHA & GNANAMANICKAM, 1990)] have been isolated from several sources , including the host plant, suppressive soils, and as spontaneous, rough avirulent mutants from wild type *P. solanacearum*. It is assumed that these organisms are able to compete under conditions where the pathogen occurs. Although promising under controlled conditions, none of these were effective under field conditions.

Characteristics of an ideal antagonist

Biocontrol strategies developed in the laboratory often fail under field conditions. For instance, antibiosis is frequently effective for limiting growth of pathogens in laboratory tests, but unsuccessful in controlling disease in greenhouse assays or in field trials. It is likely that successful control of *P. solanacearum* with antagonistic microorganisms will depend on the ability of the biocontrol agent to survive under conditions that favor the pathogenic bacterium. In the soil and rhizosphere microenvironments the biocontrol agent must contend with complex biological and physical factors, including soil composition and structure, moisture and pH, all of which can influence the structure of the microbial community. In an attempt to limit the factors with which the biocontrol agent must contend, we have turned to a new approach, *i.e* the use of endophytic antagonist derived from the wild type pathogen. Thus these microorganisms will be adapted for survival in the plant microenvironment and should therefore compete favorably with the pathogen.

Endophytic antagonism

The use of such an endophytic antagonist has several advantages over use of an external antagonist. For instance, once the antagonist has established itself within the plant, it may persist throughout plant development thereby providing continous protection. The ideal candidate should retain the characteristics required for colonization and endophytic survival but should lack the ability to cause disease. It

should be able to colonize the roots, to penetrate the xylem vessels, and to multiply within the vascular tissue.

Avirulent mutants of Pseudomonas solanacearum

Spontaneous avirulent mutants have been used with limited success as biocontrol agents against bacterial wilt (KEMPE & SEQUEIRA, 1983; TANAKA, 1986). The distinguishing feature of these mutant strains is that they are deficient in production of exopolysaccharide (EPS) which is presumed to be required for virulence. These mutants were first described by KELMAN (1954) and can be readily isolated (KELMAN & HRUSCHKA, 1973).

Some of these spontaneous, avirulent mutants have been reported to multiply within susceptible host tissue upon inoculation by the stem puncture technique (AVERRE & KELMAN, 1964). However their systemic spread is limited and their populations usually decline rapidly, probably because they are readily agglutinated by plant lectins and/or bound to host plant cell walls(for a review see SEQUEIRA, 1982). It is possible that EPS permits systemic movement of the bacterium by preventing the latter mechanisms.

A strong correlation between exopolysaccharide production and virulence has been recently demonstrated with transposon-induced avirulent mutants which lie in the *ops* (COOK & SEQUEIRA, 1991) and *eps* (DENNY & BAEK, 1991) gene clusters. We have determined the structure of the major polysaccharide (ORGAMBIDE et al, 1991) which we have named X1. From analysis of several transposon-induced avirulent mutants in the *ops* and *eps* gene clusters we concluded that there is a direct correlation between aggressiveness and the quantity of X_1 produced *in vitro* and *in planta* (unpublished data). Mutants that are devoid of X_1 are completely avirulent and unable to colonize the host plant upon root inoculation. Mutants which produce a reduced amount of X_1, that is identical to that of the wild type as revealed by 1H NMR analysis, are hypoagressive.

A second type of non-virulent mutants retain wild type EPS production but lacks the ability to cause both the hypersensitive response on resistant hosts as well as the ability to cause disease on susceptible hosts. Several of these mutations are located within the 23kb *hrp* gene cluster (BOUCHER et al., 1985 & 1987). These *hrp* mutants are avirulent on young tomato plants but retain the ability to invade through the roots and to multiply within the susceptible host (TRIGALET & DEMERY, 1986). The results obtained with these *hrp* mutants with respect to biological control can be briefly summarized as followed (TRIGALET & TRIGALET-DEMERY, 1990):

- *hrp* mutants vary according to their invasiveness when roots are inoculated without artificial injury,
- there was a positive correlation between the invasiveness of the mutant and the

level of protection achieved
- none of these *hrp* mutants exhibited antibiosis against the wild type virulent strain GMI1000 suggesting that antagonism could be due to the induction of host resistance mechanisms,
- the presence of *hrp* mutants in host tissues was correlated with reduced colonization by the otherwise virulent GMI1000. However, it is likely that protection will be strain specific because some virulent strains can produce bacteriocins that inhibit growth of the mutants that we used as biocontrol agents (FREY et al., these proceedings).

Our data indicate that prior inoculation with *hrp* mutants limit disease development upon subsequent inoculation with virulent *P. solanacearum*, even under conditions favorable for disease.

Experiments with mature tomato plants

Because we were concerned that the avirulent bacteria might negatively affect fruit production, we measured fruit yield in control and in *hrp* inoculated tomato plants. These experiments were conducted according to the recommendations of the Commission de Génie Biomoléculaire (French Institutional Biosafety Committee) who restricted the trials to a contained environment on Guadeloupe (French West Indies).

The genetically engineered avirulent mutants were obtained from native, virulent strains isolated on Guadeloupe and carried no transposable antibiotic marker.

The results demonstrated (FREY et al., 1992) that the presence of *hrp* mutants in the mature host plant did not alter tomato fruit production and that the occurence of *hrp* mutants was limited to root and collar tissues. In particular the bacteria were not detected in mid stem or in fruits. This indicates the importance of considering antagonistic effects of native virulent strains on the biocontrol agent.

Discussion

These initial results, using *hrp* mutants of *P. solanacearum* for biological control of bacterial wilt of tomato, suggest that this may be a feasible control strategy under field conditions. We have demonstrated that protection by *hrp* mutants can be effective even when a heavy concentration of challenge, virulent inoculum is applied on the roots. Under natural field conditions the resident virulent inoculum will probably be less concentrated and the resulting protection may therefore be more effficient.The next phase of experimentation will involve soils that are naturaly infested with *P. solanacearum*.

Our recent results indicate that protection against bacterial wilt could originate from the induction of host plant resistance. Similar suggestions have been made by KEMPE and SEQUEIRA (1983). Some spontaneous avirulent bacteriocin-

producing strains (ABPS) are currently used with some success against bacterial wilt (CHEN & ECHANDI, 1984). We believe that *hrp* mutants are most likely to confer protection from bacterial wilt by induction of host defence mechanisms. However it should be possible to design *hrp* mutants that produce a bacteriocin with a broad spectrum activity thereby combining both mechanisms. In the latter case it would be necessary to identify the genes which are involved in bacteriocin production. If these genes are carried on a conjugative plasmid, as in the case of *Agrobacterium radiobacter* strain K84, then one would have to delete the transfer region as was done for the biological control of crown gall (JONES et al., 1988).

The broad host range plasmid that carries the small fragment of the *hrp* gene cluster, which was inactivated by insertion of the Omega cassette, should enable us to transform any virulent strain of *P. solanacearum* to avirulence, provided there exits sufficient homology within the *hrp* sequences of interest. Thus protective *hrp* mutants of *P. solanacearum* could be obtained from native strains in different geographic locations, thereby avoiding the introduction and dispersal of non-indigenous strains. Due to the strong homology which has recently been described among *hrp* gene sequences from several different phytopathogenic bacteria *(P. solanacearum*, *Xanthomonas campestris*, *Pseudomonas syringae*, *Erwinia amylovora*: ARLAT et al., 1991; GOUGH et al., accepted for publication), it may be possible to extend this approach to include other bacterial diseases of economic importance.

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Plant growth-promoting rhizobacteria, current status

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ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) increase crop yield either by reducing the deleterious effect caused by minor pathogens and/or by suppressing well-known diseases. In addition some PGPR are able to dissolve rock phosphate or to bind N_2 in the absence of symbiosis. PGPR are part of the natural suppressiveness of the soil and they are mainly responsible for the beneficial effect of solarization. They use root exudates to produce metabolites that either directly inhibit soil-borne pathogens and their deleterious effects or that increase the resistance of the plant against disease. There is the possibility to improve the performance of PGPR by modifying their genome. The influence of PGPR and their active compounds on other components of the soil ecosystem and ground water is not well-known. It is difficult, therefore, to assess the risk of introducing genetically modified strains or highly effective wild-types into large areas. However no adverse effect has been reported up to now.

Key words: biocontrol, plant-beneficial bacteria, soil-borne plant pathogens, antibiotics, growth substance, HCN, siderophore, induced resistance, genetically modified microorganisms, risk assessment, soil, *Pseudomonas fluorescens.*

INTRODUCTION

Soil-borne pathogens cause important yield reduction in protected and unprotected crops. Such damage has increased steadily in the last decades. Pesticides are seldom effective in soil and growing concern for the environment restricts their application. The use of root-colonizing microorganisms to protect plants is a promising option for the future. Many bacterial strains have been shown to have this ability in greenhouse and field experiments. They are termed **PGPR** (**P**lant **G**rowth **P**romoting **R**hizobacteria). A few PGPR promote plant growth in the

absence of pathogenic microorganisms because of their ability to dissolve rock phosphate or to bind N_2 in the absence of symbiosis (e.g. *Azospirillum*); others probably help the germination of seeds or produce plant growth regulators. However, direct growth promotion has not been observed in the field. Most of the work done with PGPR concentrates on *Pseudomonas* strains because their obvious biocontrol effect can be analysed and possibly increased by molecular genetic methods. Moreover, *Pseudomonas* is easy to grow in the laboratory and cheap to produce on a large scale. Several reviews deal with *Pseudomonas* as PGPR (e.g. SCHIPPERS *et al.*, 1987; WELLER, 1988; DÉFAGO & HAAS 1990; GUTTERSON, 1990; LOPER & BUYER 1991; LUGTENBERG *et al.*, 1991, DOWLING *et al.*, 1992; KEEL, 1992; LEMANCEAU, 1992). This short paper will focus mainly on results obtained in our laboratory.

BIOLOGICAL ACTIVITY

Resident populations of *Pseudomonas* are part of the natural disease suppressiveness of soils and of suppressiveness induced by monoculture or solarization (STUTZ *et al.*, 1986; WELLER, 1988; DÉFAGO & HAAS, 1990; GAMLIEL & KATAN 1991). *Pseudomonas* strains reduce disease incidence caused by root pathogens and, in a few cases, protect systemically leaves and stems against air-borne pathogens when added to seeds, soil or substrates for hydroponic culture at concentrations of 10^{11} to 10^{15} cfu per ha (DÉFAGO *et al.*, 1987; WÜTHRICH & DÉFAGO, 1991). One strain might suppress more than one disease and protect more than one crop. For example, strain CHA0 of *P. fluorescens* suppresses diseases caused by *Gäumannomyces graminis* on wheat, by *Thielaviopsis basicola* on tobacco, by *Fusarium oxysporum* on tomato and by *Pythium ultimum* on different crops (DÉFAGO *et al.*, 1990; FUCHS & DÉFAGO, 1991; WÜTHRICH & DÉFAGO, 1991); strain CHA0 is also able to protect systemically. While strain CHA0 colonizes only the root system of tobacco plants, it reduces also the number and diameter of necroses caused by the virus TNV on leaves (MAURHOFER, unpublished results). Under field conditions, i. e. in different locations and/or different years, the performance of the same biocontrol agent tends to be variable (STUTZ *et al.*, 1985, 1989). This problem of site, environment or cultivar specificity has hampered commercial application (KLOEPPER *et al.*, 1988; DIGAT *et al.*, 1990). The reproducibility of the beneficial effect can be increased by the use of mixtures of PGPR or of PGPR with other beneficial microorganisms (FUCHS & DÉFAGO, 1991; LEMANCEAU, 1992).

MECHANISMS

It is generally assumed that *Pseudomonas* strains with biocontrol ability colonise plant roots and use root exudates to synthesize metabolites which either inhibit the growth and activity of pathogens or trigger systemic resistance of the plant against diseases. A *Pseudomonas* strain can produce more than one extracellular compound in batch culture (table 1).

Table 1: Secondary metabolites produced by *Pseudomonas fluorescens* strain CHA0 and their role in disease suppression

Metabolites	Properties	Effects on disease suppression in gnotobiotic systems*	References
Pyoverdine	siderophore	none	Keel *et al.*, 1991
Indoleacetate	growth hormone	none	Oberhänsli *et al.*, 1991
HCN	biocide	+ in some cases	Ahl *et al.*, 1986 Voisard *et al.*, 1989 Keel *et al.*, 1989
2,4-Diacetyl-phloroglucinol	antibiotic, herbicide	+ in some cases	Keel *et al.*, 1990,1991, 1992 Maurhofer *et al.*, 1992
Pyoluteorin	antibiotic, herbicide	?	
Pyrrolnitrin	antibiotic	?	
Monoacetyl-phloroglucinol	antibiotic	?	
Salicylate	inducer of resistance	?	

* + = positive effect; ? = effect not known

These compounds can be chemically characterized and their role in disease suppression can be assessed by testing non-producing mutants. When a mutation results in a reduction of disease suppression, the relevant genomic fragment can be cloned to demonstrate complementation of both the production of the compound and the ability to suppress disease. The natural genetic instability of *Pseudomonas* can impede this analysis by introducing cryptic mutations (OBERHÄNSLI & DÉFAGO, 1991). Moreover the loss of production of a

metabolite might also allow the bacteria to overproduce another; this might occur particularly in the rhizosphere where the amount of nutrient is limited.

The following mechanisms were proposed to explain the biocontrol effect of *Pseudomonas*: antibiosis, degradation of pathogenic toxins, competition for iron between the biocontrol agent and the pathogen, induced systemic resistance. The role of antibiosis and of the degradation of pathogenic toxins are clearly proven. Several genes which are part of the suppressive ability of the *Pseudomonas* were identified, and some were cloned and sequenced (e.g.: genes for the degradation of fusaric acid; for the production of fungitoxic compounds HCN, phenazine-1-carboxylic acid, 2,4-diacetylphloroglucinol and one gene named *gac*A which affects the global regulation of several metabolites in the stationary growth phase, TOYODA *et al.*, 1988; VOISARD *et al.*, 1989; THOMASHOW & WELLER 1988; KEEL *et al.* 1992; VINCENT *et al.*, 1991; LAVILLE *et al.*, 1992). It should be kept in mind that more than one mechanism may operate to suppress a given disease and that suppression of different plant diseases or of one disease under different environmental conditions by one particular strain of *Pseudomonas* may involve different mechanisms.Interestingly, most of the active metabolites produced by biocontrol *Pseudomonas* strains are also produced by *Pseudomonas* which have no beneficial effect. We hypothesise that the main differences between these two groups are when, where and how much of these metabolites are produced.

PERSPECTIVES

PGPR have no adverse effect on humans and animals; they have the real potential to maintain and restore soil fertility by controlling soil-borne pathogens. The development and implementation of PGPR in agricultural practice needs (i) improvement of strains by classical or genetic engineering methods to increase the consistency of their effect in field; (ii) a better knowledge of the ecological impact of the added PGPR in order to increase their effect and to assess the risks of their deliberate release; (iii) a set of protocols and regulations for release approval based on real risks (COOK, 1992). If these points are realised there is no doubt that PGPR and other biocontrol microorganisms can reduce the damage caused by soil-borne pathogens and so achieve a significant step forward in the agriculture of the twenty-first century.

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The evaluation of the effect of abiotic soil factors on actinomycetes selected as potential antagonists to plant pathogenic microorganisms

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Abstract

Several experiments involving the effects of the application of a mineral fertilizer and variations in soil moisture have been evaluated with regard to their influence on the bacterial flora of root-free soil and the rhizosphere. Only fluorescent pseudomonads were significantly influenced by the fertilizer, possibly due to the stimulation of their metabolic activity by nitrogen. In no experiment could moisture fluctuations be found to be in any way detrimental to bacterial populations. Even when significantly low levels of moisture were achieved for short periods no effect was found on actinomycetes.

Key words: actinomycetes, fluorescent pseudomonads, metabolism, moisture, nitrogen, pH, total bacteria.

Introduction

In spite of our present day knowledge and advancement along numerous scientific fronts, any significant breakthrough in the biological control of plant disease is still lacking. Any comparison of what has been achieved during the last half century with the amounts of time, effort and money that have been invested into this problem is completely disproportionate. Rather than continuing in the same way, the author takes the view that a step backwards to gain more insight into why things have gone wrong will in the long term mean less time in reaching ones goal.

To evaluate the best chance that any potential antagonist may have in the field, the use of *in vitro* and *in vivo* systems are unavoidable. However, any development of such methods should, a) take into consideration the biology of the antagonist - pathogen - host plant relationship, and b) evaluate the

influence naturally and artificially occurring factors may have on this relationship.

In order to examine the significance of these factors it was necessary to first make a choice of plant, pathogen and possible antagonist. Based on earlier rhizosphere work of NEAL *et al.* (1970) and more recent studies of MILLER, (1990); MILLER *et al*, (1989; 1990a), the relationship between wheat lines sensitive and resistant to *Cochliobolus sativus* and potential actinomycete antagonists is being investigated. In many experiments, plants sometimes have to be maintained in soil for their whole life cycle. In environmental cabinets this can take up to 9 weeks and during such an experiment the nutrient status of the soil, pH and moisture could vary to such a degree that observations of the microbial activity would be distorted.

In this paper some results on such factors will be discussed.

Material and Methods

All pots contained 2 kg root-free loamy sand (initially pH-H_2O 5.9 with a bulk density of ca. 1.2 g/cm^3) and were kept in a climate cabinet for up to 9 weeks at 20°C (R.H. ca. 70%) during the light period, and 15°C (R.H. ca. 85%) during the dark period (MILLER *et al.*, 1990a). Pots designated for wheat were sown, one plant per pot for each week (all experiments were conducted in triplicate) with asceptically germinated seedlings of line C-R_5B (MILLER *et al.*, 1989). A mineral fertilizer (MERCKX *et al.*, 1987) was used in all pots (N ≡ 140 mg/kg soil) except control series.

A soil moisture of 15% was generally achieved except in cases in which the experiment was designed to inflict dryer or irregular conditions. Details with regard to sampling methods, dry weights, moisture determination and bacterial enumeration (total bacteria, fluorescent pseudomonads and actinomycetes) has been published (MILLER *et al.*, 1990a). Data were subjected to an analysis of variance.

Results and Discussion

Maintenance of a sufficiently fertile soil to support wheat plants for experiments of up to 9 weeks was provided by the addition of a mineral fertilizer (MERCKX *et al.*, 1987). This was only added once at the beginning of each experiment before seedlings were planted. Although the rhizosphere

microflora can be indirectly influenced by fertilization (TURNER et al., 1985), little was known about a possible direct effect of mineral fertilizer on soil bacteria. In one experiment, therefore, this effect was first studied on fluorescent pseudomonads and actinomycetes of root-free soil.

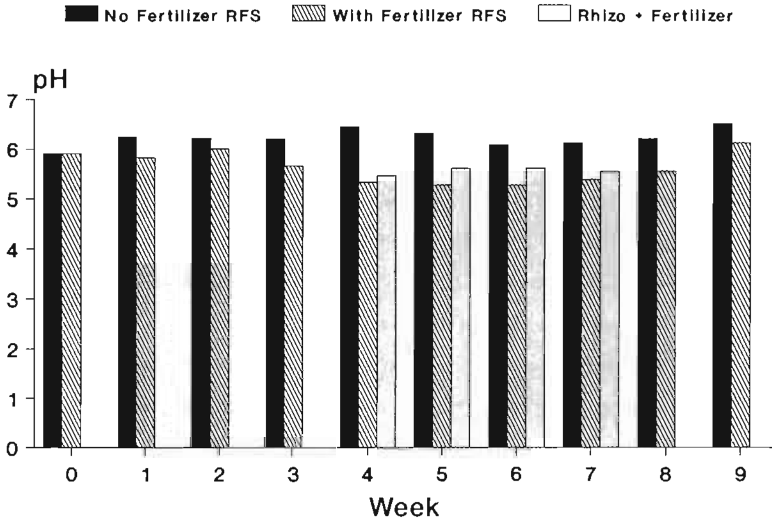

Fig. 1. The mean weekly pH of root-free soil from fertilizer amended and unamended pots. The pH rhizosphere soil amended with fertilizer is shown for weeks 4 - 7.

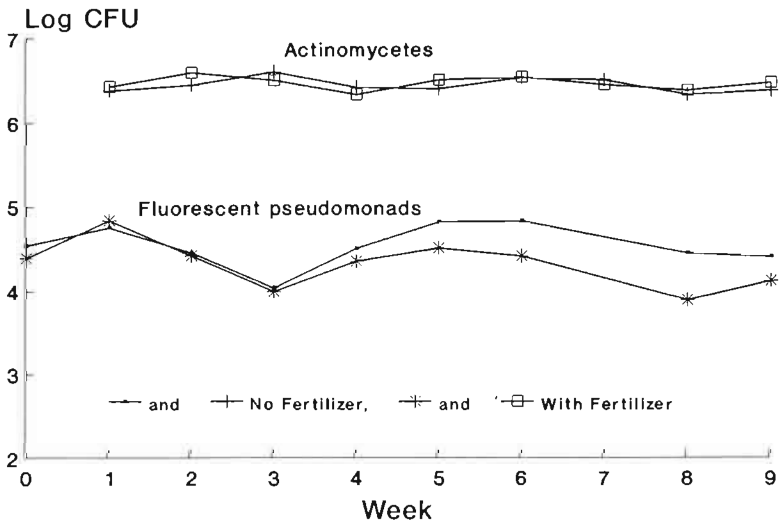

Fig. 2. Means of bacterial numbers counted each week in triplicate (Log c.f.u.) in non-fertilizer and fertilizer amended root-free soil.

A fall in pH after the 2nd week in fertilizer amended soil, although significant, was small and recovered by the 9th week. As samples were taken at the same depth, the changes could be a reflection of the migration of ions through the layer due to daily watering. Examination of the rhizosphere pH during the 4 -7 week period indicates that plant exudation may be responsible for a partial compensation of this drop (Figure 1).

On analysis of the bacterial counts, no significant differences could be found between either the total numbers of bacteria from amended and unamended soil (not shown) or between the actinomycetes counted from amended or unamended soils (Figure 2). The fluorescent pseudomonads, on the other hand, showed a significant decrease in numbers in fertilizer amended soil ($P<.001$ over the whole sampling period, $n= 53$). This became obvious after the 3rd week

Although the reduction in fluorescent pseudomonad numbers occurred at the same time as the relatively small pH drop, pseudomonads usually have been found to grow well at a lower pH common in the rhizosphere of young developing roots (unpublished results). LILJEROTH *et al.* (1990) and MILLER *et al.* (1990b) suggested that nitrogen may directly be involved in bacterial development. TURNER *et al.*, 1985 and SMILEY, 1978 also suggested that nitrogen may influence the decline of *Pseudomonas* spp. On careful examination of our results it was noted that a more rapid increase in numbers of all bacteria occurred within the first two weeks in fertilizer amended soil followed then by a fall in numbers. For the total bacterial population and for the actinomycetes this did not appear to lead to any detrimental effect for the remainder of an experiment. However, in the case of fluorescent pseudomonads, the increase in mineral nitrogen may have lead to a more effecient utilization of the limited available carbon source in the soil (LILJEROTH *et al.*, 1990). This elevated metabolic activity would mean a sudden depression of suitable nutrients available for further fluorescent pseudomonad survival.

Actinomycetes have long been known for their potential antagonism, but some reports lead us to believe that numbers may be elevated under dry conditions (LABRUYERE, 1971) and decrease under wet conditions (VRUGGINK, 1976). In our experiments water schedules normally resulted in moisture levels not lower than 11%, ca. pF 3.0 .

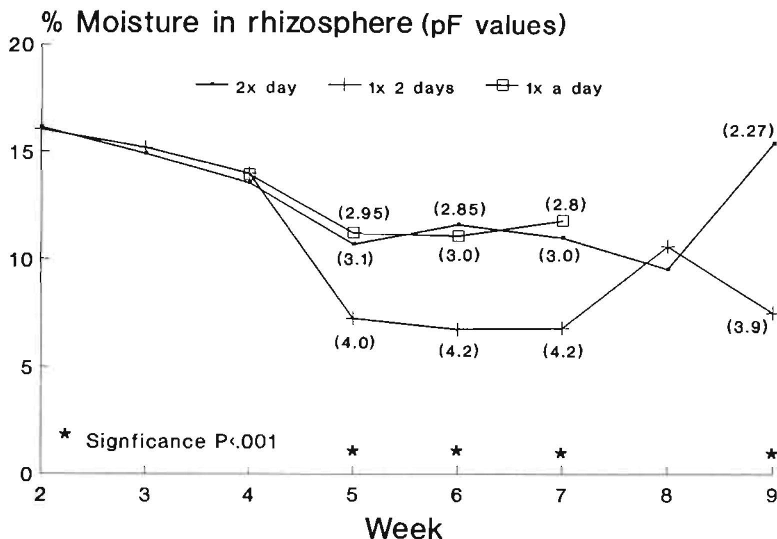

Fig. 3. Mean weekly moisture percentages of the rhizosphere resulting from three different water regimes. pF values given in parenthesis.

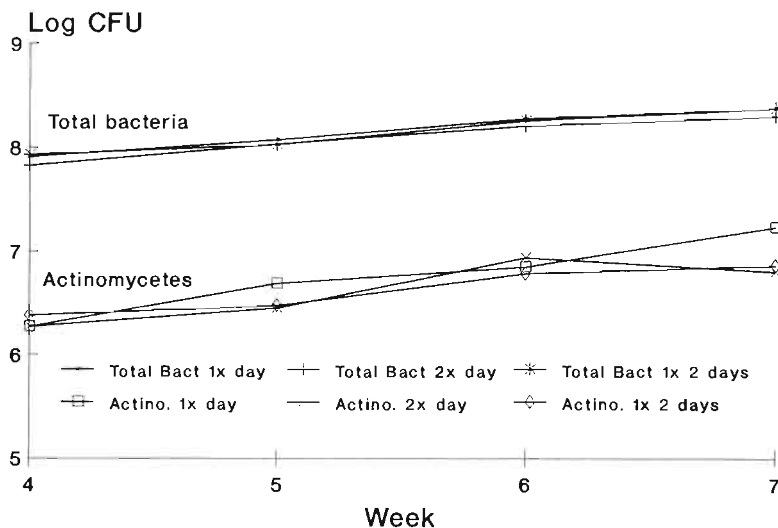

Fig. 4. Mean total bacteria and actinomycete counts (Log c.f.u.) determined from the 4th to 7th week for three different water regimes.

A significant increase in pF to ≥4.0 could only be maintained between the 5th and 7th week with a watering regime of once in two days (Figure 3). This was not sufficient, however, to significantly influence the total numbers of bacteria or the actinomycetes. WATSON and WILLIAMS (1974) reported that they could find no clear quantitative differences of actinomycete populations in sandy soils between a moisture regime of pF 1.0 and pF 2.3.

We must conclude, therefore, that it may be also true for a pF of $\geq$4.0, for short periods at least.

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Biological control of *E. lata* by an antagonistic strain of *B. subtilis*

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ABSTRACT

An isolate of *Bacillus subtilis* from grapevine wood inhibited in vitro growth of *Eutypa lata*, the causal organism of dieback in grapevines. Malformation of hyphae of *E. lata* occurred in the presence of *B. subtilis*. At a concentration of $\geq$ 0.8mg/ml, an antibiotic substance in an ethanol extract from *B. subtilis* totally inhibited germination of ascospores of *E. lata*. Spraying a suspension of the bacterium on pruning wounds before inoculation with ascospores of *E. lata* significantly reduced infection as compared with unsprayed, inoculated controls. Thin-layer chromatography of crude antibiotic extract showed five bands, two of which inhibited mycelial growth of *E. lata*.

Keywords: antibiosis, *Vitis vinefera*

Die-back of vines in South Africa has been known since 1881. Matthee & Thomas (7) reported in 1977 that the apricot pathogen *Eutypa armeniacae* was associated with vines die-back in South Africa. Rappaz (9) indicated that *E. armeniacae* is a synonym of *Eutypa lata*.

The principal method of entrance of *E. lata* is through pruning wounds, especially on wood older than two years. Due to this way of infection, it is very difficult to control this disease effectively. In order to control it effectively, every pruning wound must be covered with a protective agent which is an extremely laborious and expensive method. Biological control methods were subsequently investigated.

Various *Bacillus* spp are antagonistic to plant pathogenic fungi and bacteria (1,2). Several *B. subtilis* isolates from healthy trees were antagonistic to *V. dahliae* in maples (4) A. *B. subtilis* strain antagonistic to *Ceratocystis ulmi* was also reported (3). In this paper, we report on an isolate of *B. subtilis* from grapevine (*Vitis vinefera*) wood, which was antagonistic to *Eutypa lata* the cause of die-back of grapevines in South Africa.

MATERIALS AND METHODS:

The ability of *B. subtilis* to inhibit *E. lata* was tested by placing a loopful of bacterial culture at a distance of 10mm from actively growing mycelium of the fungus on PDA. Afterwards, plates were incubated for 7 days at 25°C and examined for an inhibition zone. The bacterial isolate which was used in all experiments was identified by the API 50 CHB system (SA Montalieu, Vericieu, France) and verified by the methods of Harrigan and McCance (5) and Sneath (10).

EFFECTS OF BACTERIA ON MYCELIUM AND ASCOSPORES OF *E. lata*.

Hyphal morphology:

E. lata was grown in CDB (Czapek Dextrose Broth) in petri dishes by transferring a mycelium plug (7 mm) from a PDA culture to the centre of a petri dish containing 15 ml broth and incubated at 25°C. After mycelium growth reached a diameter of about 10mm on the surface of the liquid, each petri dish was inoculated with a loopful from a three day old CDB culture of *B. subtilis* and incubated at 25°C for a further 3 days. Mycelium strands at the edge of the colony of each fungus were removed, placed onto a microscope slide and examined microscopically for hyphal abnormalities.

Ascospore germination:

Ascospores of *E. lata* were obtained by washing pieces of vine wood containing perithecia for 15 minutes under running tap water. The top of the perithecia were cut through with a sterile scalpel and the contents of ten perithecia placed separately in 5ml sterile distilled water in McCartney bottles and shaken well. Spores were counted with a haemacytometer and diluted to 1 x 10^4 spores/ml.

The antibiotic substances produced by *B. subtilis* was obtained from a 4d old CDB culture of *B. subtilis* incubated at 25°C, by extraction with 80% ethanol (McKeen, Reily & Pusey, (8). The antibiotic substance in 80% ethanol was added to petri dishes just before the agar was poured to provide concentrations of 0,0; 0,5; 0,6; 0,7; 0,8; 0,9 and 1,0 mg/ml (m/v). Six plates were used for each concentration and control plates received only 150 ul 80% ethanol. A few drops of an ascospore suspension 10^4 spores/ml of *E. lata* were spread over the agar by means of a bent sterile glass rod. After 24 hours incubation at 25°C a total of 100 ascospores, were examined for germination.

Field test for control of *E. lata*:

A 4-year old Riesling vineyard trellised to a Perold system was used; wounds were made on 2 year old wood by pruning. Treatments were benomyl 50WP at 10 g/l, benomyl (10 g/L) in mineral oil plus *B. subtilis* 10^8 cells/ml, antibiotic extract of *B. subtilis* (2 mg/ml), suspension of *B. subtilis* in CDB, and control (distilled water). Two additional trials of the same experiment were conducted in 1989, except that mineral oil was omitted and a treatment of *B. subtilis* + benomyl (0,5 g/L) was used.

Each wound was inoculated with 100 ul of a spore suspension (5 x 10^3 spores per milliliter) of *E. lata* 4 hour after treatment, and wounds were covered with aluminum foil to prevent dessication. Wounds varied between 10 and 15 mm in diameter. Nine months after inoculation, cane pieces were cut 25 mm below the inoculated wound. These pieces were quartered lengthwise and placed on PDA after surface-disinfestation in 0,5% sodium hypochlorite for 5 minutes. After 5 days at 24°C, isolates were examined for typical mycelial growth of *E. lata*.

Thin-layer chromatography:

The antibiotic substance in 80% ethanol was applied as a band onto $20cm^2$ silica gel plates with a fluorescent indicator. Plates were developed in ethanol: water (2:1 v/v) and the bands marked under UV light. The different bands as well as areas between them were separately scraped off, powdered in a mortar and pestal and resuspended in 80% ethanol. These extracts were tested for inhibition of mycelial growth of *E. lata* by visually evaluating the inhibition effect.

RESULTS:

Effects of bacteria on mycelium and ascospores of *E. lata*

Mycelium morphology. After introduction of *B. subtilis* to CDB, little additional growth of *E. lata* was observed. Hyphal tips of the fungus became malformed, and hyphae were thickened and vacuolar compared with hyphae in the absence of the bacterium. Many swellings occurred in the hyphae or at the tips of the hyphal strands, whereas normal hyphal walls were smooth with no swellings or vacuolation. No lysis of hyphae was observed.

Ascospore germination. Ascospores of *E. lata* did not germinate in the presence of *B. subtilis*, whereas an average of 68% germination was observed in the control after 24 hours. The mean percentage germination of ascospores of *E. lata* in controls without antibiotic extract was 70%. Germination was reduced sharply (b = -0.8386) when the extract was added to the medium. Germination of ascospores in which 0.5, 0.6 and 0.7 mg/ml of the antibiotic substance was added to the medium was 4.3, 3.3 and 1.3%, respectively; whereas no germination occured at or above a concentration of 0.8 mg/ml. (Table 1).

Table 1: Germination of *Eutypa lata in ascuspores in different bacterial toxin concentrations*.

Concentration (mg/ml)	Germination (%)
0,5	4,3
0,6	3,3
0,7	1,3
0,8	0

Field tests on control of *E. lata*:

Infection of wounds with *E. lata* during 1988 was rather low (23%). During this period, only the antibiotic substance failed to give significant suppression, whereas only *B. subtilis* gave 100% suppression of the fungus (Table 2). In 1989, *B. subtilis*, *B. subtilis* + benomyl, and one test with the antibiotic substance significantly suppressed the fungus as compared with the water control.

Table 2: **Influence of different treatments on pruning wound infection by *Eutypa lata***

	Treatment	Wound infection (%)[a]		
		1988	1989 a	1989 b
1.	Control (H_2O)	22,9	39,2	45,0
2.	Benomyl (10g/l)	4,0 x	27,5	29,2
3.	*Bacillus subtilis* (10^8 cells/ml) + benomyl + Mineral oil	3,0 x	--	--
4.	Bacterial toxin (Extracted) (2mg/ml)	5,0	12,5 x	22,5
5.	*B. subtilis* suspension in CDB	0,0 x	4,1 x	3,1 x
6.	*B. subtilis* + benomyl (0,5g/l)	--	1,6 x	3,2 x

[a] Means within columns followed by the letter x differ significantly from the control at P = 0.05 according to the least significant dÖ

Thin-layer chromatography:

Chromatography of crude antibiotic extract on silica gel plates yields five bands under UV light, with R_f values 0.47, 0.55, 0.65 and 0.75. Extractions from each of these bands and areas between bands showed that only the bands with R_f 0.55 and 0.59 inhibited mycelial growth.

DISCUSSION:

The isolate of *B. subtilis* from dieback-affected grapevine wood produced at least two antibiotic substances in vitro that inhibited mycelial growth and ascospore germination of *E. lata*. Mycelial malformation was observed and probably due to the antibiotic substances interfering with normal growth processes. The vacuolar appearance of the mycelium and ascospores probably was due to antibiotics produced by the bacterium, which may have penetrated and caused protoplasmic dissolution. (6)

Biological control was obtained with the antibiotic substance(s) in the 1989a test, but is was relatively less effective compared with *B. subtilis* and *B. subtilis* + benomyl. Suppression with benomyl was insignificant in the 1988 and 1989b tests, but *B. subtilis* + benomyl gave significant control.

The use of *B. subtilis* as a biocontrol agent against *E. lata* may be an economical way to suppress the disease. The form in which the bacterium can be applied commercially needs further research.

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Biological control of the bacterial ring rot disease of potato (*Clavibacter michiganensis* subsp. *sepedonicus*) using a naturally occurring antagonistic bacterium

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ABSTRACT

Bacteria were isolated from potato tuber surfaces and selected for antagonistic activity toward *C. michiganensis* subsp. *sepedonicus*, the causal agent of bacterial ring rot of potatoes. A two-step screening procedure involved isolation of colonies that inhibited growth of *C.m. sepedonicus* in a four-layer agar plate technique, and subsequent selection in greenhouse tests using tissue-cultured potato plantlets inoculated with *C.m. sepedonicus* and antagonistic isolates. In the greenhouse bioassay, the protective effect of antagonistic bacteria was determined after plantlets had grown for 2-3 weeks by testing for the presence or absence of detectable levels of the pathogen by the enzyme-linked immunosorbent assay (ELISA). An isolate (16C) was selected on the basis of the initial screening procedure as having the greatest potential for biocontrol. It was tested in a field experiment by inoculation on tuber seed-piece surfaces prior to inoculating the tubers with *C.m. sepedonicus* using a ring rot contaminated knife. Inoculation with 16C increased plant stand and fewer plants from treated seed pieces developed ring rot symptoms. Isolate 16C was tentatively identified as *Arthrobacter protophormiae* on the basis of whole cell fatty acid profile. A hybridoma cell line was developed that produced a specific monoclonal antibody to this isolate. The monoclonal antibody reacted with 16C in immunofluorescence and in double sandwich ELISA in which antigen was trapped with a polyclonal antiserum. Specificity of the monoclonal antibody was determined by testing it against other antagonistic bacteria isolated from different potato samples. Strain 16C was detected by ELISA and immunofluorescence with the monoclonal antibody on progeny tubers harvested from field plants grown from 16C-inoculated seed pieces. The antagonist was detected on tubers even after a 6 month storage period.

INTRODUCTION

The bacterial ring rot disease of potato, caused by *Clavibacter michiganensis*

subsp. *sepedonicus,* is an important disease in several potato growing regions. The primary method for controlling the disease is the use of ring rot-free seed tubers (De Boer and Slack 1984). However, the pathogen may be present in symptomless plants and tubers from which it can spread during planting and harvesting (Nelson 1982). Consequently, procedures have been developed to test for the presence of the ring rot pathogen in symptomless plants and tubers (De Boer *et al* 1989).

Secondary sources of inoculum may also exist in potato fields. Recently it was shown that sugar beet roots (Bugbee *et al* 1987) may harbour *C. m. sepedonicus*, and it was also shown to be present in weed rhizospheres (Zizz and Harrison 1991). Pathogen-free seed could be contaminated from these sources as well as through inadvertent contact with an infected seed lot or contaminated farm equipment. The objective of this study was to determine whether naturally occurring bacteria antagonistic to *C.m. sepedonicus* could be used to prevent infection of ring rot-free seed lots.

It had been observed that ring rot occasionally did not develop in plants grown from seed pieces infected with bacterial ring rot (De Boer and McNaughton 1986). Furthermore, it was observed that the presence of soft rot bacteria in the seed piece inhibited development of ring rot in inoculated seed pieces (unpublished, De Boer). Rapid decay of the seed piece possibly prevented *C.m. sepedonicus* from being translocated to the stem growing from the seed piece. By increasing the population of antagonistic bacteria on the seed piece it may be possible to prevent low populations of *C.m. sepedonicus* from secondary infection sources to become established in a tuber lot.

MATERIALS AND METHODS

Isolation of antagonists. Bacteria antagonistic to *C.m. sepedonicus* were isolated from the surface of potato tubers, suspended in one quarter strength Ringer's solution and 100 µl of several dilutions added to 3 ml of molten soft YGM medium (yeast extract, glucose, mineral salts medium with 0.6% agar). The molten agar was then poured into a plate containing 15 ml of YGM medium with 1.5% agar. After the soft medium solidified a 3 ml aliquot of sterile soft YGM medium was layered overtop the bacteria-containing layer. The plates were incubated overnight at 28 C and then a final 3 ml-layer of soft medium with about 10^9 colony forming units (cfu)/ml of *C.m.*

sepedonicus strain R8 was poured into the plate. Plates were incubated for 3-5 days at 23 C. *C.m. sepedonicus* formed a lawn of micro-colonies in the top layer and zones of inhibition appeared at locations immediately above bacteria growing in the center layer that produced substances antagonistic to it. The antagonistic bacteria could then be isolated directly by culturing them from inoculum taken through the inhibition zones. After retesting the isolated strains for antagonistic activity the bacteria were stored in glycerol at -80 C.

Screening for biocontrol potential. Biocontrol potential of antagonistic bacteria was determined in micropropagated plantlets planted into soil in a greenhouse. Nodal cuttings of a ring rot susceptible potato cultivar, Red Pontiac, were grown and rooted in glass jars containing Murashige and Skoog medium. Plantlets were removed from the medium and then inoculated by cutting the roots back about one-third and immersing the roots sequentially into a suspension of 10^8 cfu/ml of antagonistic bacteria and in 10^6 cfu/ml of *C.m. sepedonicus.* Root-inoculated plantlets were planted in a soil mix and grown in a greenhouse at about 21 C with a 16 h photoperiod using supplementary light from sodium vapour lamps. Uninoculated plantlets and plantlets inoculated with the ring rot pathogen alone were used as controls. After two weeks, stems of the plantlets were tested for the presence of *C.m. sepedonicus* by ELISA using monoclonal antibody 1H3 (De Boer *et al* 1988).

Field test of an antagonistic bacterium. One isolate of antagonistic bacteria selected for further testing was inoculated onto whole seed pieces by immersing them into a suspension of 10^8 cfu/ml for 30 min. After drying the tubers overnight they were inoculated with ring rot by cutting them into seed pieces with a knife dipped into a slurry of 0.4% agar containing 10^9 cfu/ml of *C.m. sepedonicus* strain R8. Uninoculated tubers and tubers inoculated with *C.m. sepedonicus* alone were used as controls. Seed pieces were planted in the field, 24 seed pieces per plot, with 4 replications in a randomized block design. During the growing season, plant stand, the proportion of plants with ring rot symptoms, and the proportion of plants positive in an ELISA test for ring rot were recorded. At harvest the number of tubers with ring rot symptoms and asymptomatic infections were determined.

Identification and detection of an antagonistic bacterium. The antagonistic bacterium showing the greatest potential as a biological control agent was identified by Microbial Identification System (Newark, DE) using the gas chromatographic profile

of the cellular fatty acids. A rabbit polyclonal antiserum was produced to the antagonistic bacterium by immunization with whole, glutaraldehyde-fixed cells. A monoclonal antibody was produced to the same strain using standard cell fusion techniques to develop a hybridoma cell line which produced antibodies of the desired specificity. The monoclonal antibody was used in indirect immunofluorescence and in a double antibody sandwich ELISA in which the polyclonal antibody was used for trapping the antigen and the monoclonal antibody as the specific probe.

RESULTS AND DISCUSSION

Almost 100 isolates which were antagonistic to *C.m. sepedonicus* were obtained from the initial isolation plates. One strain, labelled 16C, consistently prevented infection of the micropropagated plantlets with ring rot in 4 repeated tests (Table 1). This strain was tentatively identified as *Arthrobacter protophormiae* on the basis of fatty acid analysis. A hybridoma cell line was selected, which produced an antibody useful in both immunofluorescence and ELISA tests. No cross-reactions were observed when it was tested against *C.m. sepedonicus* and 20 other antagonist tic strains.

Table 1. ELISA values as a measure of BRR infection in micropropagated potato plantlets inoculated with BRR and isolate 16C.

	Mean ELISA Value		
Test	R8 and 16C	R8 alone	Uninoculated
1	0.133	0.610	0.126
2	0.118	0.370	0.085
3	0.061	0.120	0.048
4	0.134	0.432	0.096

In the field plot experiment the number of plants that developed from the 16C treated seed pieces was equal to the uninoculated control treatment and slightly greater than the number of plants from the seed pieces inoculated with ring rot alone (Fig. 1). The differences were not significant at the 95% probability level, however. The number of plants that expressed ring rot symptoms and the number of plants positive for ring rot in ELISA was significantly lower in the plots with the 16C treatment in comparison to the ring rot control treatment.

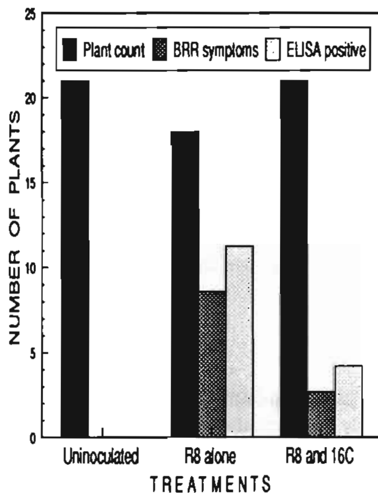

Figure 1. Development of bacterial ring rot (BRR) disease in potato stems grown from seed pieces inoculated with isolate 16C in comparison to control treatments.

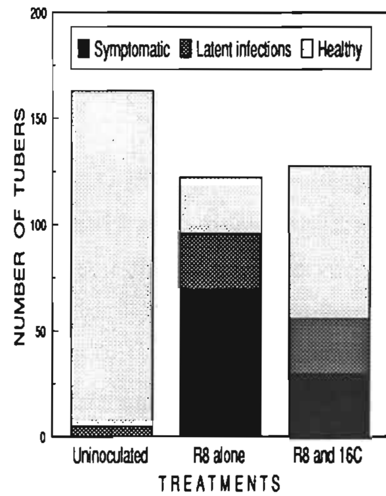

Figure 2. Bacterial ring rot in progeny tubers from plants grown from seed pieces inoculated with isolate 16C in comparison to control treatments

The average number of progeny tubers produced in the uninoculated control plots was 20% greater than in the treated plots but the difference was not significant at the 95% level (Fig. 2). The 16C inoculation, however, significantly reduced the number of tubers visibly infected with ring rot, while the number of tubers with symptomless infections did not differ between treatments.

Although complete control of ring rot was not achieved in this experiment, significant reduction in disease incidence occurred. The level of ring rot inoculum used in this experiment was several orders of magnitude greater than the inoculum load expected from secondary sources. Moreover, the 16C inoculum was applied to the tuber periderm only, while the ring rot inoculum was applied to the cut tuber surface, so antagonist and pathogen were not inoculated at the same site. Use of a carrier to enhance survival of the biocontrol bacterium may also increase effectiveness of the biocontrol. It is anticipated that if the level of ring rot inoculum is low, infection might be prevented with inoculation of 16C.

For a biocontrol agent to be effective it must survive well in association with the host. Hence serological procedures were used to determine whether 16C was present on the progeny tubers from the field plot experiment. Significant levels of 16C

were detected by immunofluorescence and ELISA on the surface of progeny tubers from plots inoculated with the antagonist but not in tubers from other plots, after the tubers had been kept for 6 months at 4 C after harvest (Table 2). The absence of bacteria reactive to the 16C monoclonal on tubers from plots not inoculated with the antagonist confirmed the specificity of the antibody. The presence of 16C on progeny tubers from plants grown from 16C-inoculated seed pieces showed that it survives well on potato tuber surfaces, as would be expected from a bacterium isolated from that niche.

Table 2. Detection of isolate 16C on progeny tubers from plants grown from uninoculated and 16C inoculated seed pieces with a monoclonal antibody in immunofluorescence (IF) and ELISA.

TEST	Uninoculated			Inoculated with 16C		
	Tuber 1	Tuber 2	Tuber 3	Tuber 1	Tuber 2	Tuber 3
IF	-	-	-	-	+	+
ELISA	0.044	0.023	0.052	0.046	0.788	0.453

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Characterization of the diversity of rhizosphere fluorescent pseudomonads populations with lipoprotein (OPRI) gene and siderophore polymorphism

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Rhizosphere fluorescent pseudomonads are often involved in antagonism against root plant pathogens, in stimulation of mycorrhization or in plant growth promotion. Besides this wide range of biological activities, the diversity of total populations of these widespread rhizobacteria is rarely taken into account in ecological studies.

A collection of fluorescent pseudomonads was isolated from wheat root necrosis caused by Gaeumannomyces graminis var. tritici, the take-all pathogen. All the strains belonged to the different biotypes of Pseudomonas fluorescens and P. putida, and to some intermediate biotypes as characterized with classical taxonomical tests.

The lipoprotein OPRI gene (1) was amplified by PCR with Taq polymerase and gave a single 0.24 kb DNA product for each strain. This result confirms the conservation of this gene within the pseudomonads of rRNA group I (2). Exhaustive digestion of PCR products with PvuII, Hae III or SphI showed a polymorphism for these restriction sites. RFLP analysis on total bacterial DNA digested with PvuII using PCR products as probes allowed the establishment of the different strains in a limited number of patterns.

Fluorescent pseudomonads produce fluorescent siderophores (pyoverdines) which are thought to cause antagonism. These metabolites are constituted of a chromophore and a linked polyamino acid chain. The various pI of the lateral chain with the aminoacids composition allowed the separation of purified siderophores after isoelectrofocusing on a polyacrylamide gel. An agarose overlay containing CAS solution (3) for detection of siderophores revealed the same groups. This last experiment confirms the presence of siderophores. On this basis, different forms of siderophores were observed for each strain and different patterns were described among this population. Some typical

associations between RFLP patterns and siderophore patterns were observed (Table 1).

These two criteria, namely the lipoprotein gene and the siderophore structure polymorphisms offer complementary informations for the analysis of the diversity of fluorescent pseudomonads populations in the rhizosphere.

Table 1: Characterization of the most representative strains of *Pseudomonas fluorescens* isolated from wheat root necrosis caused by *Ggt*: biochemical grouping (L: levane, G: gelatine, T: trehalose, D: denitrification), pyoverdines pI, restriction sites after PCR amplification of lipoprotein gene (PCR/LPP), RFLP analysis of genomic DNA digested with PvuII (RFLP/LPP).

Strain	Biotype	Biochemical grouping L G T D	pI Pyoverdines	PCR/LPP HaeIII	PCR/LPP PvuII	PCR/LPP SphI	RFLP/LPP (PvuII)
AS 11	II, IV	+ + + +	7, 8.1	+	-	+	5 kb
AS 22	II, IV	+ + + +	7, 8.1	+	-	+	5 kb
AS 39	II, IV	+ + + +	7, 8.1	+	-	+	5 kb
AS 46	III	- + + +	6.8, 8	+	-	+	3 kb
AS 1	V	- + + -	7	-	+	+	1.7, 0.7 kb
AS 25	V	- + - -	7	-	+	+	1.7, 0.7 kb
AS 35	V	- + - -	7	-	+	+	1.7, 0.7 kb
AS 37	V	- + - -	7	-	+	+	2, 0.7 kb
AS 48	V	- + - -	7	-	+	+	5 kb
AS 21	?	- - - +	7.4	-	-	+	1.2 kb
AS 30	?	- - - +	7.4	-	+	+	>10 kb
AS 14	?	+ + - +	7.4	-	-	+	2.2 kb

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In vitro production of antibiotics by strains of *Erwinia herbicola* selected for biocontrol of *Erwinia amylovora*

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INTRODUCTION

Fire blight of rosaceous plants, caused by Erwinia amylovora, is particularly suitable for biological control since the major phase of infection occurs via floral stigmatic or nectary surfaces (WILSON et al., 1989) so that effective application of antagonists can be limited both in time and space.

Various naturally occurring phylloplane bacteria have been used as antagonists, including Pseudomonas syringae (WILSON et al., 1992) and white or yellow pigmented strains of Erwinia herbicola (RIGGLE & KLOS, 1970; BEER et al., 1987 and WILSON et al.,1990). The activity of these antagonists at the plant surface may involve a number of factors, including production of antimicrobial compounds (antibiotics, siderophores or bacteriocins) and site or nutrient competition.

There has been considerable interest in recent years in the production of antibiotics by Erwinia herbicola and the role that these may have in planta. WODZINSKI et al.,(1990) have shown that the in vitro production of antibiotics by this organism correlates with the biological control of fire blight in orchards. ISHIMARU et al., (1988) and WINKELMANN et al., (1981) have isolated antibiotics from liquid culture medium and used a variety of physico-chemical techniques to demonstrate that they are polypeptides.

The studies carried out in this report, on white pigmented strains of Erwinia herbicola, had the following aims:

1. Examination of antibiotic production in vitro in relation to a range of test organisms (spectrum of activity).
2. Isolation and characterisation of antibiotic compounds.
3. Raising polyclonal antibodies to selected antibiotics with a view to determining whether these compounds are produced in situ on the plant surface.

(1) SPECTRUM OF ANTIBIOTIC ACTIVITY

The extent to which antibiotics produced by isolates of *Erwinia herbicola* are able to inhibit a range of test microorganisms *in vitro* was examined by conventional plating procedures. Plugs from cultures of *Erwinia herbicola* were placed on agar plates freshly sprayed with lawns of various microorganisms. The observation of inhibition zones allowed the spectrum of activity of different strains to be determined (Table 1).

Table 1 Differential inhibition of test microorganisms by strains of *Erwinia herbicola*

	Serratia marcescens	*Bacillus cereus*	*Pseudomonas syringae*	*Escherichia coli*
EhWhL 7	13	9	12*	8*
EhWhL9	-	-	-	13
EhWhL13	10	-	-	11
EhWhL68	7	13	-	12*
EhWhL71	-	-	13	-
EhWhF18	6	-	40	19
EhWhF18.Nal	-	-	-	10
EhNL18	16	-	-	9
EhNL18.Nal	14	-	-	12
EhNL19	-	-	-	10
EhYC37	-	-	-	14
Eh252	14	-	-	17

Strains of *Erwinia herbicola* are listed in left-hand column. * indicates multiple inhibition zones. All measurements given are inhibition zone diameters (mm), mean of three replicates.

The results, which are consistent and repeatable, show that antibiotic production by live cells of different *Erwinia herbicola* strains growing on minimal agar medium have varying effects on different test organisms.

(2) ISOLATION AND CHARACTERISATION OF ANTIBIOTICS

The production of antibiotics was partially characterised by thin layer chromatography (TLC), with further analysis using High Performance Liquid Chromatography (HPLC). Preliminary studies on membrane-partitioned populations had shown that antibiotics were able to diffuse through Visking tubing with a M.W. cut-off of 10-12,000, indicating a molecular weight of less than this value.

a. Thin layer chromatography

MATERIALS AND METHODS.

Strains of *Erwinia herbicola* were grown in the

liquid medium of WINKELMANN et al. (1981), modified by the substitution of glycerol as a carbon source. Cell-free supernatants, sampled at different stages in the growth cycle, were concentrated 10-fold *in vacuo* and examined by TLC. Chromatography plates were developed in methanol and inhibitory compounds visualised by the application of a spray of *Escherichia coli* strain 0111, followed by the pigment aesculin (LUND & LYON, 1975).

RESULTS.

Two major antibiotics were identified from the thin layer chromatogram of each *Erwinia herbicola* strain examined. One of these migrated in the solvent (Rf value 0.5), the other remained on the baseline. The antibiotics were also visualised using Ninhydrin after acid hydrolysis *in situ* and an Aniline phosphate spray, revealing respectively that the migratory antibiotic was a peptide and the baseline spot a carbohydrate. Although these two major types of antibiotic were common to all strains examined, differences were noted in the size of the spots and exact position of the peptide antibiotic on the TLC plate.

The production of both peptide and carbohydrate antibiotics varied considerably during the growth cycle, as shown for strain EhWhL9. In this isolate the peptide compound showed a sharp rise at the end of log phase, with both antibiotics exhibiting a marked decline during stationary phase.

b. *High Performance Liquid Chromatography*

MATERIALS AND METHODS

Purified samples of the mobile antibiotic were obtained from strain EhWhL9 by preparative TLC and examined using HPLC, using a Tris/HCl-Methanol solvent system. Absorbance of the eluant was measured using a Diode Array detector.

RESULTS

The HPLC trace shows an initial solvent peak, followed by a sequence of antibiotic constituent peaks - suggesting that the antibiotic has been separated into a series of subunits (or has broken down in the column). Each of these constituents has a typical protein uv spectrum, confirming the polypeptide nature of the compound.

(3) *IMMUNOLOGICAL STUDIES*

Polyclonal antibodies were raised to the purified polypeptide antibiotic, carbohydrate antibiotic and crude filtered supernatant derived from *Erwinia herbicola* strain EhWhL9.

MATERIALS AND METHODS

Since the peptides concerned are small (c.10,000MW) molecules, probably of low antigenicity, these were

complexed to cholesterol, phosphatidylcholine and the glycoside adjuvant Quil-A to form immunostimulating complexes (ISCOMS), following the method of MOREIN et al. (1989). The protocol for this technique involved coupling purified antibiotic (obtained by preparative TLC) with a purified protein derivative of tuberculin (PPD) prior to ISCOM formation. Purified ISCOMS were injected into rabbits pre-conditioned with a BCG vaccination. A booster was given after four weeks, and test bleeds made after a further two weeks.

RESULTS

The appearance of a negative stain preparation of immunostimulating complexes (conjugated with purified peptide antibiotic) under the transmission electron microscope (TEM) is shown in Fig. 1. These electron microscope images have the cage structure typical of ISCOMs, confirming that conjugation with the PPD-antigen has taken place.

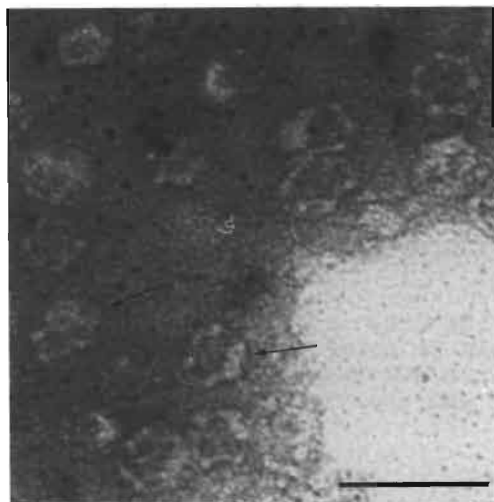

Fig.1 TEM negative stain preparation of ISCOM particles (arrows). Scale - 50nm.

Antisera obtained from the rabbits have so far been tested against the original antigens (using ELISA), and shown to give a clear immune reaction in each case. The specificity of these will be determined in future studies, with the objective of antibiotic location at the plant surface by immunofluorescence and immunogold labelling.

DISCUSSION

Several workers have discussed the role of antibiotic production by Erwinia herbicola within the context of biological control of fire blight. ISHIMARU (1985) has examined the effect of strain sensitivity of the pathogen on the different levels of control achieved using purified antibiotic compounds, and both ISHIMARU and WODZINSKI et al.

(1990) note that factors other than antibiotic production are also involved.

The spectrum of activity of the antibiotics described here is fairly wide, and includes the inhibition of both non-phylloplane and phylloplane organisms. The presence of multiple inhibition zones with some test organisms indicates different spectra of activity for the various antibiotics involved.

Studies on the formation of antibiotics in liquid culture reveal that they are formed chiefly in early stationary phase, and broken down later on. The late production of antibiotics may reflect their production at the onset of nutrient deficiency, when such compounds could play an important role in the competitiveness of a population on the phylloplane surface.

The polypeptide identity of the TLC-mobile antibiotic is in agreement with the data obtained by other workers, including ISHIMARU (1985), BEER et al. (1987) and WINKELMANN et al. (1981), who have made various proposals for the precise molecular structure. The production of antibodies to these antibiotics opens up the possibility of immunolabelling of these compounds on the plant surface, with future studies on the role of antibiotic production and activity *in planta.*

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Investigations on the biology of *Streptomyces* sp. K_{455} and the properties of produced antibiotic complex

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INTRODUCTION

Bacterial diseases of plants are widespread, and they cause great losses of crop. Together with widely used chemical preparations (BOGATZEVSKA, 1989 ; DEVASH *et al.*, 1980 ; SCHAAD, 1980 ; DIXON, 1981), antibiotics are also applied (TSILOSANI *et al.*, 1981 ; PYKE, 1984) for plant protection.

This report describes investigations on *Streptomyces* sp. K_{455} and on the properties of its product, an antibiotic complex.

MATERIAL AND METHODS

Streptomyces sp. K_{455} is isolated from Kenya's soil samples.

A morphological description of the culture is done as shown in "International *Streptomyces* Project" (1966) and also by using Gausae's Guide for Actinomycetes Identification (1983). Cultural variability is studied on 17 media, which are recommended by KUZNETSOV (1973).

Utilization of carbon sources is examined by the method of PRIDHAM and GOTTLIEB (1948).

The culture is grown in submerged and aerobic conditions at a temperature of 28°C$\pm$ 1.

The antimicrobial activity of the antibiotic complex and of two of its components are determined by the method of agar diffusion (LANCINI and PARENTI, 1982). This complex is obtained as a crude product by extraction with low alcohols from the culture mycelium. Its heterogeneity is investigated by thin layer chromatography and a separation of the two components carried out on a column with aluminium oxide carrier and n-butanol as eluent. Antibiotic solubility of the components is determined by chromatography (MERCK, Art. 5550, Aluminium oxide 60 F 254 neutral (Type E)) in a variety of solvents recommended by OMACHI and CEVCIK (BLINOV and HOHLOV, 1970) and in different buffers.

As a model to examine the activity of the antibiotic complex *in vivo* naturally infected soybean seeds with page are used. The seeds are treated with water-alcohol solution (8:2) of antibiotic in different concentrations. The extent of seeds damage is determined at the 5th stage of Bogatzevska's scale (1979) and an index of disease is calculated according to Mc Kinney's formula (1923).

RESULTS

The investigation is connected with the determination of cultural characteristic of the strain. It is observed that the colour of aerial mycelium is grey and the back side of colonies varies from pale yellow to brown on most of the used media (table. 1).

TABLE 1 : Cultural characteristics of Streptomyces sp. K_{455}

Medium	Growth	Reverse side of colony	Aerial mycelium
Yeast extract-malt extract agar (ISP 2)	Moderate	Yellow-brown	Grey
Oatmeal agar (ISP 3)	Good	Dark brown	Dark grey
Inorganic salts-starch agar (ISP 4)	Good	Brown	Dark grey
Glycerol-asparagine agar (ISP 5)	Moderate	Brown-yellow	Grey
Mineral agar (Gausae)	Good	Colour less to yellowish	Dark grey
Organic agar 2 (Gausae)	Moderate	Colour less to yellowish	Pale grey

The morphological characteristics of the strain are examined with both optical and electron microscopes. The aerial mycelium is branched monopodially and formed spiral chains of spores with 1 or 2 spirals per chain. Spores have a warty surface (see fig. 1 and fig. 2).

The culture shows a great variability and it is well defined on Krasilnicov's media (KUZNETSOV, 1973) - it divides into six morphological types with different antimicrobial activity.

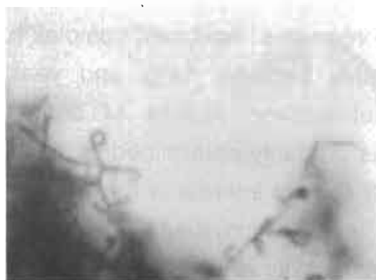

Fig. 1 Micrograph of aerial mycellum of *Str. sp.* K_{455}

Fig. 2 Electron micrograph of spores of *Str. sp.* K_{455}

Capability to utilize carbon sources is shown in table 2.

Table 2 : Carbon utilization of Streptomyces sp. K_{455}

Carbon source	Growth	Carbon source	Growth
L-Arabinose	±	hamnoseR	+ +
D-Xylose	±	Raffinose	+ +
D-Fructose	+	D-Mannital	+ +
D-Glucose	+	I-Inositol	+ +
Sucrose	+	Cellulose	±

± weak growth; + growth; ++ well defined growth

Based on the results shown in tables 1 and 2 and according to additional results (data not shown), *Streptomyces* sp. K_{455} is identified as *Streptomyces hygroscopicus* K_{455}

Our investigations show that the antibiotic complex consists of three components, which are respectively named A, B and C. The first two components, which are colourless, are isolated with the help of column chromatography, with a maximum of absorption for component A : 206 nm and for component B : 206 nm and 253 nm.

Two of them dissolve in low alcohols (methanol, ethanol, n-butanol) and esters (ethyl acetate), but they are not soluble in acetone, ether, petrolium ether and halogenated carbo-hydrogens (chloroform, tetrachlormethane). These two components are insoluble in the pH interval 2.0-9.0.

Antimicrobial activity of the antibiotic complex and the isolated components (A and B) was studied against different test-micro-organisms. Antibiotic complex has activity against Gram-positive and Gram-negative bacteria, fungi and yeasts. Isolated components have mainly antibacterial activity. Activity of antibiotic complex, as a whole, against fungi and yeasts is probably determined by the 3rd component, C, and partially by component A. Of special interest is the activity of antibiotic complex against *Psgl, Pst* and *Ps. car.*, which cause diseases of soybean, tomatoes and carnations. That is why its activity is tested against *Psgl in vivo*. The seeds are treated with different concentrations of antibiotic complex - interval of 0.5-3.5 g per 1000 g of seeds and the index of disease is calculated for each dose. The results are shown on table 3.

Table 3 : Soybean seeds treatment and index of disease

Antibiotic dose/1000g seeds	Index of disease (%)
Control	12.8
0.5g	7.6
1.0g	3.1
1.5g	1.8
2.0g	1.8
2.5g	3.3

The optimum dose of antibiotic is based on the calculation of an index of disease : it is 1.5 g antibiotic/1000 g seeds. There is no negative effect on germination of all treated seeds.

DISCUSSION

Actinomycetes strain *Streptomyces* sp. K_{455} is isolated from Kenya's soil samples. It produces a complex of antibiotics which has activity against phytopathogenic bacteria : *Psgl, Pst* and *Ps. car.* It is shown that this complex has a high effectiveness and low toxicity in the decontamination of soybean seeds from *Pseudomonas syringae* pv. *glycinea* in laboratory tests.

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The expression of antagonism of potential actinomycete antagonists to be used in the control of *Erwinia carotovora* subsp. *atroseptica* and *Erwinia chrysanthemi* pathogenic to seed potatoes. I. Development of an *in vitro* test system

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Abstract

The development of an *in vitro* test system, to be used for the reliable selection of potential antagonists to *Erwinia carotovora* subsp. *atroseptica* and *E. chrysanthemi*, requiresa soil ecological approach. Culture media, prepared from unheated potato and soil extracts, are used to provide an *in vitro* nutrient condition expected to exist at the potato surface. Suggestions are made for the procedures of isolation and storage of potential antagonistic organisms until they have been correctly assessed.

Key words: actinomycetes, antibiosis, biological control, *Erwinia*, media, nutrition, pH, potato, soil, temperature.

Introduction

The control of potato diseases caused by *Erwinia carotovora* subsp. *atrosetica* (Eca) and *E. chrysanthemi* (Ech) is presently being investigated with reference to post green harvesting of seed potatoes. Most screening procedures for possible antagonistic organisms have traditionally depended on the use of *in vitro* methods, similar or somewhat modified, which have been employed in the pharmaceutical research industry for the last half century. However, in agriculture, *in vitro* methods which may provide the possiblity of screening thousands of isolates, generally have proved to be unreliable in their prediction of biological control. In recent years the emphasis has been placed on the development of rapid *in vivo* screening methods (KLOEPPER, 1991).

In our research we hope to effectuate control of disease with antagonists selected from actinomycetes indigenous to the soil in which the potatoes are

cultivated (MILLER *et al.*, 1990; NEAL *et al.*, 1970). In order to increase the predictability of such potential isolates, it has been found necessary to review the various stages used in selection and preparation of these bacterial isolates. This does not mean the rejection of *in vitro* methods but rather the adoption of an approach in which we utilize our knowledge of the different biotic and abiotic activities which may occur at the surface of the potato. In the first step of our *in vitro* method, the prevailing temperature, pH and nutrition have been regarded as the main factors to be taken into consideration, although, it is not our intention to minimize the importance of other factors such as soil moisture, soil composition and soil texture. It should be made clear, however, that only limited results are available for presentation in this paper due to the early stage of our investigations.

Materials and Methods

Chitin Oatmeal Agar - COA (see MILLER *et al.* 1989) was used as control medium for actinomycete growth and Potato Dextrose Agar (PDA) as control for the growth of *E. c. atroseptica* and *E. chrysanthemi*. Other media used will be fully described in a different publication (in preparation). Basically, however, SEA is a soil extract agar prepared without the use of heat and sterilized by gamma radiation. Similarly $\#4^{Rad}$ is a medium produced from potato peel without heating and is also sterilized by gamma radiation. The pH (H_2O) of root-free soil and of soil adhering to potato surface was measured. Incubation of both *Erwinia* isolates and the actinomycete isolate were conducted at 20°C and 25°C.

Initial isolation of actinomycetes from soil washed from the surface of potatoes was made on a modified SEA medium based on earlier work (MILLER *et al.* 1989; POLSINELLI and MAZZA, 1984). This method as well as the use of membrane filters for replicate plating and the double layer technique used for the measurement of antagonistic (antibiotic) activity will be published in detail (in preparation). Isolation is conducted at 18°C with membrane filter and 24°C after removal of filter. Actinomycete isolates required for further investigation are first placed in liquid nitrogen for 5 minutes and then stored at −20°C. Subculturing is reduced to a minimum and the measurement of antagonist activity is measured only at 18°C.

Results and discussion

A comparison of bacterial growth at 20°C is shown in Table 1. Results very similar to these were obtained when the experiment was repeated two weeks later. Parallel experiments were also conducted at 25°C (not shown) but the results only indicated a more rapid growth. The cultivation of both *Erwinia* isolates on SEA^{Rad} was repeated several times at both temperatures but growth either failed or was barely visible. This result was not unexpected as most Gram negative soil bacteria related to the Enterobacteriaceae require high nutritional values for growth (MILLER, 1990) unlike actinomycetes which may grow on various media according to their minimum requirements. In the latter case SEA^{Rad}, as shown in Table 1, supported the actinomycete isolate almost as well as on COA, on which actinomycetes are known to grow well (MILLER *et al.*, 1989). SEA^{Rad} has proved on numerous occasions in this laboratory to be a suitable medium for the support of actinomycete growth (unpublished results). The pH of root-free soil in which potatoes were grown was found to be 5.2. An unsupplemented potato peel agar ($\#4^{Rad}$) at a pH of 6.1, which was measured from the soil adhering to the potato surface, supported a good growth of all three isolates, *E. chrysanthemi* growing more abundantly.

Table 1. Comparative degree of growth of an actinomycete and two *Erwinia* isolates after 10 days incubation at 20°C.

Culture media[a]	Actinomycete	*E.c. atroseptica*	*E. chrysanthemi*
COA pH 7.0	+ + + +	n.d.	n.d.
PDA pH 6.9	+ +	+ + + +	+ + + +
SEA^{Rad} pH 5.2	+ + +	±	±
$\#4^{Rad}$ pH 6.1	+ + +	+ + +	+ + + +

Note: [a], Rad = sterilized by gamma radiation; n.d., not done; ±, doubtful growth; +, poor growth; + +, moderate growth; + + +, good growth; + + + +, abundant growth.

Although it is often essential to obtain good growth of bacterial isolates in the laboratory, it does not necessarily reflect either the nutrient condition of the natural environment of the bacterium or its metabolic state. Sugars, proteins and most other additives used in the preparation of culture media

may be totally foreign to bacteria situated in their natural habitat. Similarly heated extracts of soil or plant material will render a product used in some culture media which does not occur in nature and may incorrectly influence an organism's metabolic systems. In our research we have attempted to create a system in which the nutritional quality is comparable to that found under natural conditions.

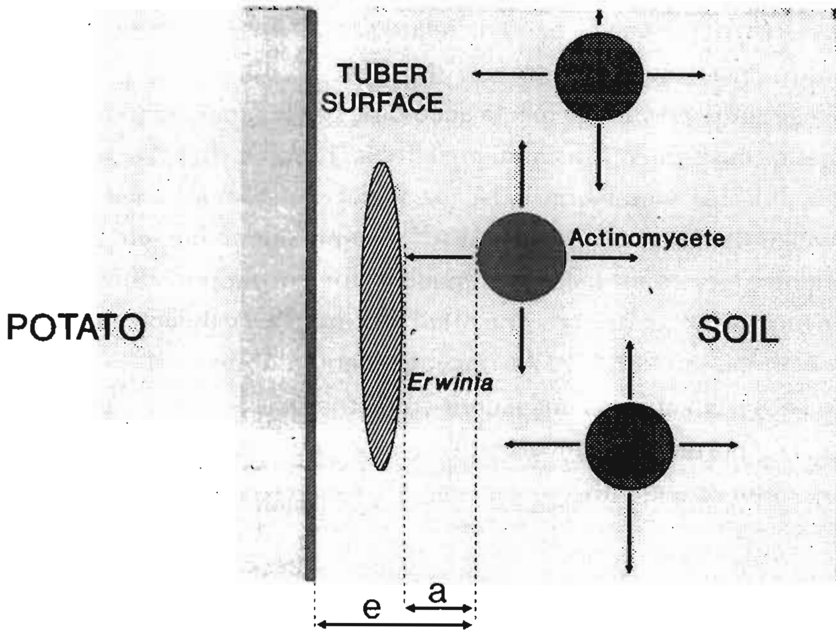

Fig. 1. **A diagrammatic impression** of a pathogenic bacterium (*Erwinia* spp.) close to the potato surface and adjacent actinomycete cells which may have antagonistic potential. **a = antagonistic influence; e = effective nutrient gradient.**

In Figure 1, we can envisage two basic nutrient sources: a) the soil which may be low in nutrient value but relatively stable, and b), the potato which, according to its own activity and condition, functions as a variable nutrient source. These combined sources, depending on various other biotic and abiotic factors in the soil at the potato surface, will be responsible for a condition to develop which could be described as a "variable nutrient gradient". This nutrient gradient influences not only the growth and activity of the pathogenic bacteria that may be present but all microorganisms that may be situated within its sphere of influence. Antagonistic

organisms, e.g. actinomycetes, therefore, may also be stimulated to grow and their antagonistic mechanisms activated. Should the range of their antagonistic influence be great enough, they could then either bring about an effect on the pathogenic activity of the pathogen or even destroy it.

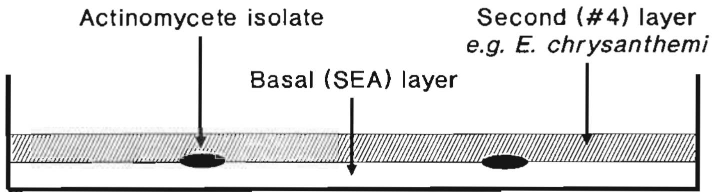

Fig. 2. Standardized double layer system used for measuring antagonistic activity at 18 °C.

On conversion of this principle to more practical terms we can envisage an *in vitro* situation (Figure 2) which consists of two layers. One layer (upper) made from potato peel extract containing either *E.c. atroseptica* or *E. chrysanthemi* and the second layer (base), a soil extract agar to maintain the actinomycete being tested. Within a relatively short time a more stable nutrient gradient will be formed by natural diffusion and bacterial activity will commence in a similar way to an activated situation under *in vivo* conditions.

Only a few but encouraging results are available at this time. When more results with this method become available during the next two years and the results compared to *in vivo* situations by careful analysis, only then can we permit ourselves to sit in judgement. Many present day scientists condemn *in vitro* methods, e.g. double and triple-layer methods (HERR, 1959; KOIKE, 1967) as being outdated, however, we are unable to except that any other method has lead to a general breakthrough in biological control. Especially those who are advocating the use of *in vivo* methods (KLOEPPER, 1991; WELLER and COOK, 1983) are utilizing techniques in the early stages of their selective procedures which still run them at the risk of developing antagonists that will only lead to meagre or no biological control under field conditions.

There is a future for *in vitro* techniques such as double-layer methods providing they are approached and devised with the eye of a biologist from a soil ecological point of view. This is equally true for every step of the way until antagonistic activity has been established. Isolation techniques, the

correct culture media for isolation and storage of potential antagonists until their use *in vitro* are being developed in this way (see Material and Methods). The same principles, lacking in most present day *in vitro* and *in vivo* research, need to be applied by all investigators.

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Longevity of bacterial antagonists in the control of fire blight of perry pears

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ABSTRACT Strains of *Erwinia herbicola* were tested for their ability to control fire blight on perry pear trees, by spray application. The longevity of these and other strains of *E. herbicola* was examined by testing for their persistence on the flowers, leaves and bark of trees. The presence of *E. herbicola* was monitored up to one year after application. The interaction of *E. herbicola* with the resident epiphytic microflora was also assessed. Additionally, the spread of applied organisms to unsprayed trees was considered.

Some strains of the applied bacteria persisted for at least 21 days on flowers, and *E. herbicola* was present on leaves at least 163 days after application, although was not detected after a year. *E. herbicola* was also present on bark for at least 163 days, but was not detected after one year. The persistence of *E. herbicola* did not appear to correlate to the *in vitro* production of inhibitory compounds by the applied bacteria.

KEY WORDS: fire blight, biocontrol, epiphytic microflora.

EXPERIMENTAL Strains of *E. herbicola* which had been selected by an immature pear fruit assay, and in some instances had also shown biocontrol of blossom and shoot blight under protected conditions (WILSON, EPTON & SIGEE, 1990), were tested for their ability to control fire blight on perry pear trees by spray application at an orchard near Taunton, Somerset, U.K. Trees (cv. Red Pear) were inoculated with *Erwinia amylovora* (10^8 cfu ml^{-1} in 1/4 strength Ringer's solution) to ensure the development of fire blight, 24h after the *E. herbicola* strains (10^8 cfu ml^{-1} in 1/4 strength Ringer's solution). Full details of these results will be published elsewhere, but a summary of the effect on the percentage of blossoms with fire blight 19 days after infection, in comparison to the infected control, is shown in Table 1. This also shows the effect on the percentage of blighted spurs 135 days after infection. The most effective strain against both blossom and spur infection was NL18.

Table 1. Percentage reduction in blossom blight 19 days after infection and spur blight 135 days after infection, due to application of *Erwinia herbicola* strains 24h before *Erwinia amylovora*

	E. herbicola strain			
	WHF18	**WHL9**	**NL18**	**NL19**
Blossom blight reduction %	26.0*	35.6*	54.1*	15.0
Spur blight reduction %	22.6	14.3	54.8*	30.1

* significant reduction in blight compared to pathogen alone (p=0.05)

Fig 1 Longevity of nalidixic acid resistant *Erwinia herbicola* strains on perry pear cv. Pine

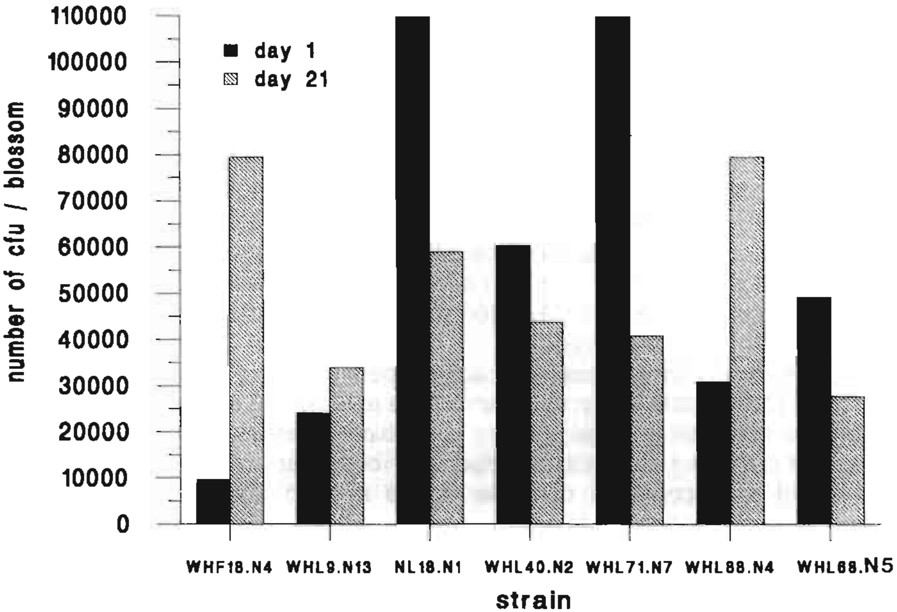
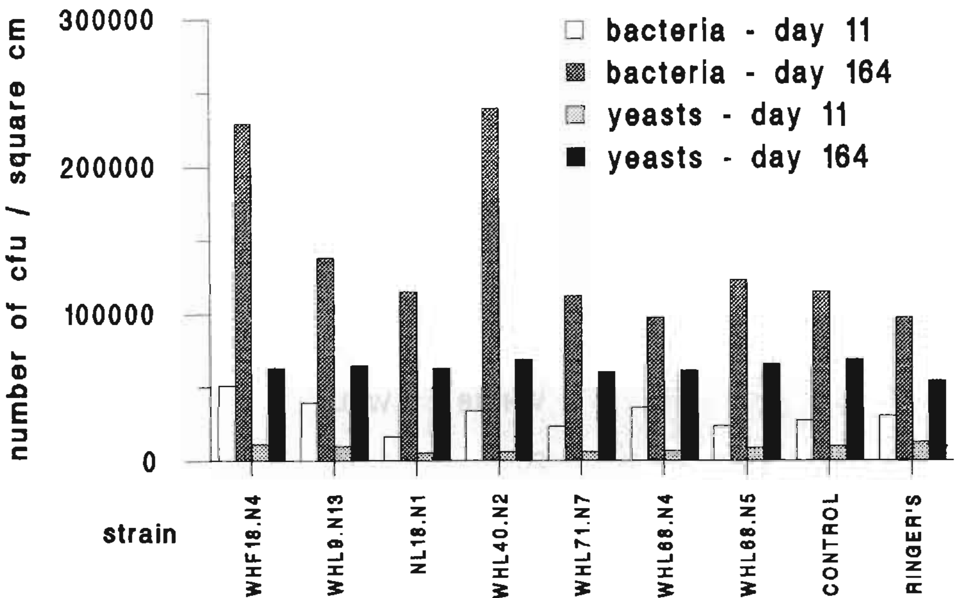


Fig 2 Epiphytic bacteria and yeasts on the phylloplane of perry pear cv. Pine, 11 and 164 days after inoculation with *Erwinia herbicola* strains

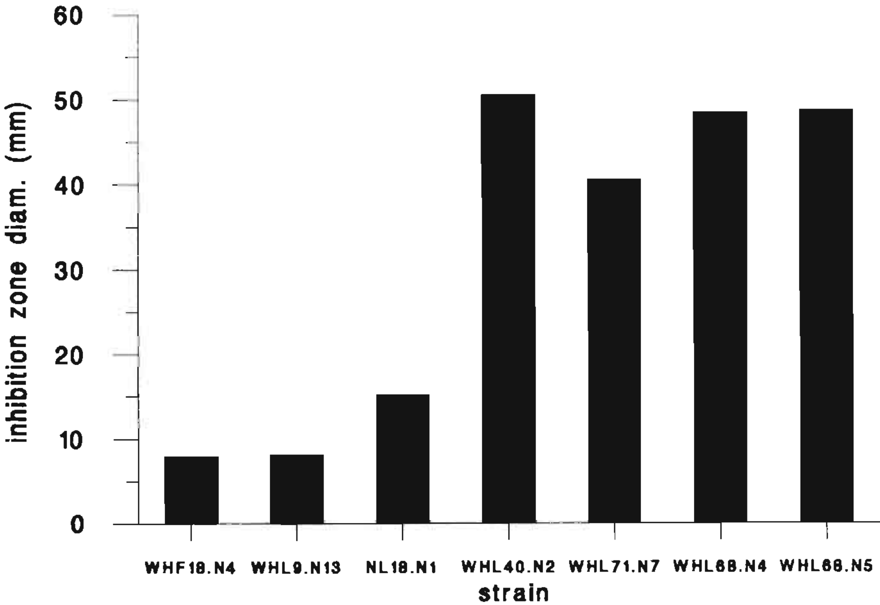


The longevity of spontaneous nalidixic acid resistant mutants of these and other strains of *E. herbicola* (applied at 10^8 cfu ml^{-1} in 1/4 strength Ringer's solution), was monitored for up to a year on the flowers, leaves and bark of trees (cv. Pine) by plating washings on nalidixic acid supplemented (100ppm) Sasser's medium (WILSON, EPTON & SIGEE, 1990). Figure 1 shows the numbers of each strain surviving on blossom after 21 days, compared to the numbers present one day after inoculation. All of the strains except WHL68.N5 were detected on leaves 163 days after inoculation. Numbers detected were too low to record accurately on leaves at this time or on bark after 157 days, but were fewer than 70 cfu cm^{-2}. The lower limit of detection was 10 cfu cm^{-2}. No isolates survived for a year at detectable levels on flowers, leaves or bark (limit of detection 30 cfu cm^{-2}).

The spread of the applied *E. herbicola* to adjacent, unsprayed trees was investigated but no spread was detected (limit of detection 30 cfu cm^{-2}).

The interaction of *E. herbicola* with the resident epiphytic microflora was also assessed. Figure 2 shows the changes occurring in populations of bacteria and yeasts on leaves, between 11 and 164 days. Comparison with the control (unsprayed) suggests that the normal seasonal variation of yeast populations was unaffected by the application of *E. herbicola*, and in general the same can be said of the bacterial populations. However, WHF18.N4 and WHL40.N2 did appear to have some effect in stimulating the general bacterial population 164 days after the *E. herbicola* application.

Fig 3 Inhibition zones produced by strains of Erwinia herbicola against E. amylovora on potato dextrose agar



There did not appear to be any correlation between the production of inhibitory compounds by the *E. herbicola* strains when tested against *E. amylovora* on potato dextrose agar (Fig. 3) and their longevity on the plant surface, or their observed influence on the microbial epiphytic flora (Fig 2).

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Biological control of soil-borne fungal pathogens by *Pseudomonas cepacia*

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Introduction

Application of bacterial antagonists of fungal pathogens for biological control of plant diseases have been widely reported. Seed coating, especially with rhizobacteria belonging to the fluorescent *Pseudomonas* group such as *Pseudomonas fluorescens* and *Pseudomonas putida* has resulted in plant growth promotion and disease suppression (Kloepper *et al.*, 1989). Recent and numerous reports have been published on the isolation and application of a non-fluorescent pseudomonad, *P. cepacia* for the biological control of soil-borne and aerial fungal pathogens (Table 1). *P. cepacia*, a soil bacterium, described earlier as a phytopathogen causing sour skin of onion bulbs (Burkholder, 1950) shows a broad spectrum anti-fungal activity against a range of fungal pathogens and is able to colonize rapidly the rhizosphere of plants such as maize, sunflower, radish, sugarbeet and pea. We review in this article the work done so far on the utilization of root-associated *P. cepacia* as an antagonist of soil-borne fungal diseases and the mechanisms involved.

Antimicrobial agents produced by *P. cepacia*

Antibiotic and siderophore production by antagonistic bacteria has been shown to be some of the important mechanisms involved in the biological control of soil-borne pathogens (Gurusiddaiah *et al.* 1986; Thomashow and Weller, 1987). Elander *et al.* (1968) found that some strains of *P. fluorescens* and *P. multivorans* (syn. *P. cepacia*) they examined produced pyrrolnitrin, a broad spectrum antifungal compound. This compound was first reported by Arima *et al.* (1964) from a

Table 1.

Examples of suppression of plant diseases by *Pseudomonas cepacia*

Strain origin	Crop	Pathogen / Disease	Reference
Soil	onion	*Fusarium* - wilt	Kawamoto & Lorbeer (1976)
			Plant Dis. Rep. 60, 189-191
Lettuce	egg plant	*Verticillium* - wilt	Homma *et al.* (1985)
			Ann. Phytopath. Soc. Japan
Lettuce	tomato	*Fusarium* - wilt	5, 543
Apple leaf	apple	*Penicillium* - blue & grey mold	Janisiewiz & Roitman (1988)
	pear	*Botrytis*	Phytopath. 78, 1697-1700
Lettuce	radish	*R. solani* - damping-off	Homma & Suzui (1989)
			Ann. Phytopath. Soc. Japan
			55, 643-652
Eastergrass	*in vitro*	maize pathogens aerial & root	Jayaswal *et al.* (1990)
			Appl. Environ. Microbiol.,
			56, 1053-1058
Pea	pea	*Aphanomyces* - root rot	Parke 1990
		Pythium - root rot	Phytopath. 80, 1307-1311
Lettuce	sugar beet	*A. cochliodes* - damping-off	Homma *et al.*, 1990
		R. solani - damping-off	
Sunflower	Sunflower	*Sclerotium* - wilt	Hebbar *et al.* (1991)
			Plant & Soil, 133, 131-140
Soil	Tobacco	*P. solanacearum* - wilt	Aoki *et al.* (1991)
			Agr. Biol. Chem. 55, 715-722
Maize	Maize	*Fusarium* - seedling rots	Hebbar *et al.* (1992)
			Soil. Biol. Biochem. in press
		Pythium - seed rots	Hebbar *et al.* unpublished

Pseudomonas pyrrocinia strain. Recently, in addition to this, *P. cepacia* strains have been shown to produce not only antifungal and antibacterial compounds such as pseudanes (Homma *et al.* 1989) and 2-keto-D-gluconic acid (Aoki *et al.* 1991) but also siderophores (Meyer *et al.* 1989). Homma *et al.* (1990) reported that although only small quantities of pyrrolnitrin were produced by *P. cepacia* strains RB425 and 1218 isolated from lettuce roots, it was more important for the suppression of damping-off in sugar beet than the pseudane antibiotics also produced. They also showed that the reference onion strain ATCC 25416 did not produce pyrrolnitrins and was not effective in suppressing the damping-off. Hebbar *et al.* (1992) have also shown that the majority of the reference soil strains of *P. cepacia* were unable to suppress *Fusarium moniliforme* infection of maize seedlings, while the maize root associated strains producing pyrrolnitrin could.

Ability of *P. cepacia* strains to colonize roots

Although antagonistic bacteria have been used extensively as seed inoculants for enhancing protection of crops from selected phytopathogens, recovery of inoculants from roots has been variable and they fail to survive and persist in the soil. Bowen (1980) has suggested characters such as high soil populations, high growth rates, resistance to antibiosis and high competitive ability as key to successful establishment by a particular microbial inoculant on the root. Studies on *P. cepacia* strains isolated from maize have shown that they are able to colonize extensively maize roots growing under different pedo-climatic conditions and are therefore potential suppressor of maize soil-borne diseases (Hebbar *et al.* 1992). Similar results have been obtained in radish (Homma *et al.* 1989), pea (Parke, 1990) and sunflower (Hebbar *et al.* 1991).

Phenotypic and genetic characterization of antagonistic and pathogenic strains of *P. cepacia*

Gonzalez and Vidaver (1979) reported that although *P. cepacia* of plant (onion) and clinical origin were similar when conventional biochemical tests were used, bacteriocin production patterns, onion maceration tests and hydrolysis of low pH pectate agar clearly differentiated them into two distinct groups. Clinical strains were negative for antibiotic production, onion maceration and pectinase activity while the plant strains were positive. Majority of the maize strains isolated showed very little maceration of onion tissue, high pectinase activity, and broad spectrum antifungal activity (Hebbar *et al.* 1992). Lennon and DeCicco (1991)

who studied the antibiotic resistance patterns and plasmid profiles of *P. cepacia* of clinical, pharmaceutical and soil origins showed that the strains with greater antibiotic resistance and most variety in size and numbers of plasmids were mainly the former two.

P. cepacia is an efficient biocontrol agent

The widespread application of biological control of plant pathogens has been hampered by the specificity of the biocontrol agents for certain soils and crops and their ability to control only one plant pathogen. However, *P. cepacia* strains seem to overcome this constraint. Production of antimicrobial compounds, high growth rates, capacity to utilize a wide range of carbon sources and also production of extracellular enzymes may be responsible for their efficient colonization of roots.

Conclusion

Evidence to date has shown that for a bacterial inoculum to be capable of inducing plant growth or suppress plant pathogens, it should be first established in the rhizosphere or the infection site. Results available so far indicate that root-associated *P. cepacia* strains are able to meet this criteria. However, clearer picture of their diversity may be obtained using rRNA:DNA hybridization and DNA restriction fragment length polymorphism so as to differentiate the benificial antagonistic strains from the pathogens.

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Protection against fire blight by an avirulent strain of *Erwinia amylovora:* possible role of the host in the interaction

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ABSTRACT

It has been previously shown that an avirulent wild strain of E. a. was able to protect a host plant against inoculation of a virulent one, when applied in suitable conditions (PAULIN, 1978). Studies were undertaken to analyse how is achieved this protection with a naturally avirulent strain (CFBP 1376). Young seedlings of apple were inoculated with avirulent and virulent strains after wounding of the youngest unrolled leaf according to different timing. Symptoms were assessed, and during 7 days population dynamic was determined for each of the two strains. Three factors suspected to have an influence on protection were studied (i) intervall between inoculation of the protective strain and the challenge test (ii) concentration of the strains (iii) avirulent/virulent ratio.
Results showed that the highest level of protection was reached when the avirulent strain was applied before the challenge inoculation and with such inoculum concentration wich gave an avirulent/virulent ratio of 10/1 associated with a concentration of avirulent strain of 10^6 cfu/leaf or more ; this suggests that a competition for sites of infection may be of leading importance . In the cases where the protection was effective the virulent strain did not multiply in the plant and which is more in most cases its population decreased together with the population of the avirulent strain ; this could mean that in addition to the site competition a plant defence response plays a role in limiting bacterial populations during the interaction.

Keywords : Erwinia amylovora, virulence, biological control, defense mechanisms.

Introduction

First attempts at biological control of fire blight were made more than 60 years ago. Various biological agents have been tested : antagonistic bacteria, bacteriophages, avirulent strains of E. amylovora, incompatible pathogens, killed bacteria...(VAN DER ZWET and KIEL, 1979)
It has been previously shown that an avirulent wild strain of E. amylovora was able to protect a host plant against subsequent inoculation by a virulent one, when applied in suitable conditions (PAULIN, 1978).
We have undertaken studies to analyse how is achieved such a protection. Three factors suspected to have an influence on protection were investigated i) intervall between the inoculation of the protective strain and the challenge test ii) concentration of the both strains iii) avirulent/virulent ratio. Moreover we have determined population dynamic of each strain (protective and challenge) in situation of protection and of non-protection.

LEVEL OF PROTECTION

Table 1-1

Inoculation of the protective strain before the challenge strain

The protective strain (1376) and the control (water) were inoculated Δt :2h or 8h before the challenge strain (1430-3.10^6 cfu/leaf)

	infected plants**
	15 days
Control (water) before 1430	
Δt : 2h	90%
Δt : 8h	60%
1376 - 3.10^7 cfu/leaf before 1430	
Δt : 2h	10%*
Δt : 8h	10%*
1376 - 3.10^6 cfu/leaf before 1430	
Δt : 2h	50%
Δt : 8h	30%
1376 - 3.10^5 cfu/leaf before 1430	
Δt : 2h	90%
Δt : 8h	40%
1376 - 3.10^4 cfu/leaf before 1430	
Δt : 2h	100%
Δt : 8h	80%

* significant difference (5%) with control
** 10 plants per treatment

Table 1-2

Inoculation of the protective strain after the challenge strain

The protective strain (1376) and the control (water) were inoculated 2h after the challenge strain (1430)

	Infected plants **	
	7 days	15 days
Control (water) 2h after 1430		
1430 3.10^4 cfu/leaf	70%	95%
1430 3.10^5 cfu/leaf	90%	100%
1430 3.10^6 cfu/leaf	70%	90%
1376 (3.10^7 cfu/leaf) 2h after 1430		
1430 3.10^4 cfu/leaf	5%*	65%
1430 3.10^5 cfu/leaf	25%	70%
1430 3.10^6 cfu/leaf	65%	80%
Control 1376 (3.10^7 cfu/leaf) 2h before		
1430 3.10^6	0%*	5%*

* significant difference (5%) with control
** 20 plants per treatment

Material and Methods

- Plant material :

  Actively growing young apple seedlings from Golden delicious (5 to leaves)

- Strains of E. amylovora :

  CFBP (Collection Française de Bactéries Phytopathogènes) 1430 (the standard strain for breeding program in France) and CFBP 1376 (a naturally avirulent strain) were used in all experiments, exept for those concerning population dynamic where 1430 Sp^r and 1376 St^r (naturally resistant mutants to spectinomycin and streptomycin, respectively) were used.

-Inoculation :

  The youngest unrolled leaf was wounded by a double cutting through the midrib (≃1mm), then inoculated with one drop (10 (ul) of bacterial suspensions (avirulent and virulent) according to different timing. 10 or 20 seedlings per treatment were used. The tests were performed in a greenhouse specially devised for inoculation with bacteria potentially dangerous for th environment.

- Population dynamic.

  Population dynamic was determined by grinding inoculated leave or whole plant in a phosphate buffer (0,2M), then plating suitable dilution on a King medium B supplemented with the appropriate antibiotic.

- Lectures

  Disease expression was assessed during 2 weeks. The number of necrosis on petiole and stem was noted.
  Population dynamic was determined at 6h, 24h, 72h, 168h after inoculation.
  Experiments were repeated at least 3 times.

Results and discussion

1) Level of protection obtained

- Influence of the concentration of the strains and of the intervall between inoculation of the protective and of the challenge strain.

Results are given in tables 1-1 et 1-2

Preliminary experiments indicated that a good control could be achieved if the strain 1376 was inoculated 2h before strain 1430 with a 1376/1430 ratio of 10/1. Our results show that the use of a lower concentration of the protective strain resulted in a decrease of the protective effect. At the optimal concentration of 3.10^7 with the challenge inoculation at 3.10^6 cfu/leaf, the lenght of the intervall between protective and challenge inoculation did not seem to play a role in the level protection. It has not been possible to study a 24h intervall between 1376 et 1430 : with our inoculation procedure, wounding of the leaf 1 day before inoculation gave an extremely low percentage of infected plants in the unprotected control, probably due to healing.

LEVEL OF PROTECTION

Table 2

Influence of the avirulent/virulent ratio

The protective strain (1376) and the control (water) were inoculated 2h <u>before</u> the challenge strain (1430)

		infected plants** 15 days
control before 1430		
water	3.10^4 cfu/leaf	75%
water	3.10^5 "	90%
water	3.10^6 "	90%
water	3.10^7 "	100%
1376 before 1430		
3.10^5	3.10^4 "	55%
3.10^5	3.10^5 "	80%
3.10^6	3.10^5 "	30%*
3.10^6	3.10^6 "	50%
3.10^7	3.10^6 "	5%*
3.10^7	3.10^7 "	25%*

* significant difference (5%) with control
** 20 plants par treatment

- Influence of the avirulent/virulent ratio

Results are given in table 2

These results show that in our conditions, protection can be obtained only when the protective strain (1376) is inoculated before the challenge test (1430), and only with an inoculum concentration giving an avirulent/virulent ratio of 10/1. Which is more it appears that a concentration of the avirulent strain of 3.10^6 cfu/leaf or more is necessary to reach a high level of protection.

The protective strain 1376 inoculated 2h after 1430 did not induced a good protective effect ; nevertheless the expression of symptoms is delayed if the concentration of strain 1376 is 100 or 1000 x higher than that of 1430.

These data suggest that a competition for sites of infection may be of leading importance in determining the success or the failure of the protective effet ; this competition seems to take place very early, during the inoculation process.

2) Population dynamic

Results are shown in figures 1 and 2

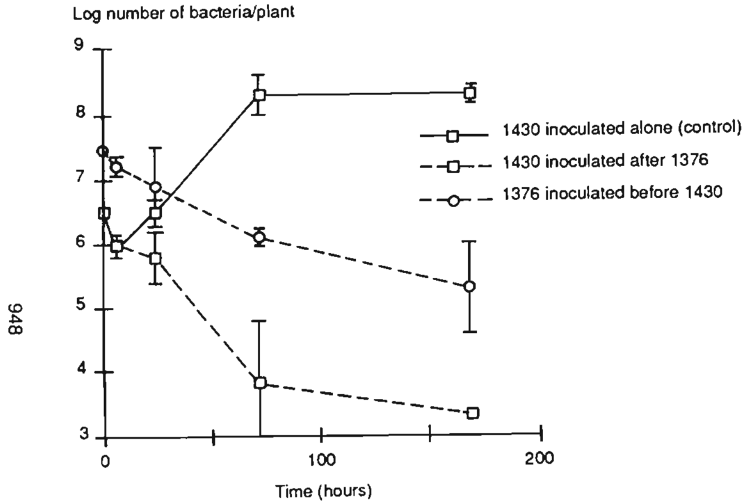
As it was expected, in all experiments, a 90 to 100% control was reached when avirulent 1376 (3.10^7 cfu/leaf) was inoculated 2h before challenge 1430 (3.10^6cfu/leaf) (figure 1). No control was obtained when 1376 was inoculated 2h after 1430, with the same respective concentrations (figure 2)

The assessment of bacterial populations showed that when the protection was effective (fig. 1) the multiplication of virulent strain 1430 was inhibited in the plant, as early as 6h after inoculation ; then its population decreased sharply to very low level (ca.10^4 cfu/leaf at 7 days). The population curve of 1376 seemed to parallell those of 1430. In some cases of effective protection, the multiplication of the virulent strain was inhibited but the decrease of its population to 10^4 cfu/leaf was only reached at 14 days. (data not shown).

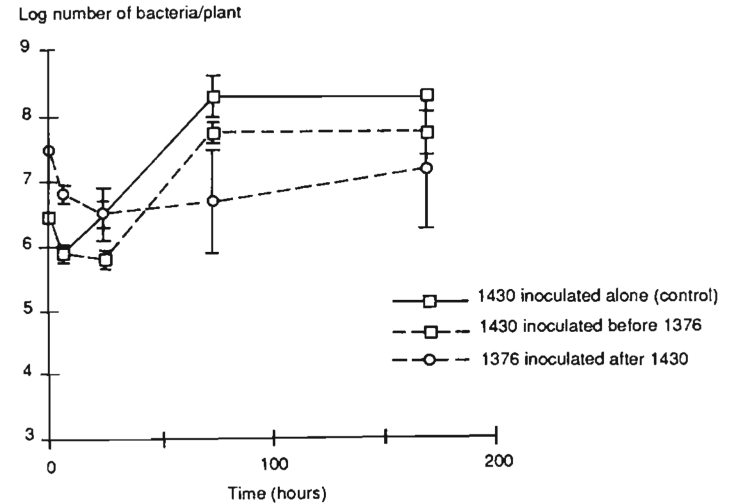
When protection did not occur (fig.2) multiplication of 1430 was not suppressed but only delayed.

The results could mean that in addition to the site competition a plant defense response plays a role in limiting bacterial populations during the interaction. If a nutritional competition cannot be totally excluded, it does not play a key role during the first hours of the interaction

POPULATION DYNAMICS



Seedlings were inoculated with avirulent strain 1376 (3.1e7 cfu/leaf) 2 hours before virulent strain 1430 (3.1e6 cfu/leaf). Each point is the mean of population of 3 plants. Bars indicate confidence intervall (5%)



Seedlings were inoculated with avirulent strain 1376 (3.1e7 cfu/leaf) 2 hours after virulent strain 1430 (3.1e6 cfu/leaf). Each point is the mean of population of 3 plants. Bars indicate confidence intervall (5%)

Conclusion

It seems therefore that, after inoculation with an avirulent strain, events leading to a situation of protection or of non protection take place very early and are likely to involve a competition for sites of infection as well as a plant defence response which inhibit the multiplication of the virulent strain.

How is modulated this possible role of the plant in the interaction ? Are others avirulents strains of E. amylovora as effective as CFBP 1376 ? Studies are in progress to investigate this point using avirulent well characterized mutants of CFBP 1430 (Barny and al, 1990) as inducers. Examining population dynamic of avirulent strains, playing the role of challengers, seems also of interest to approach mecanisms of the plant response.

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Induced resistance to Pierce's disease of grapevine by weakly virulent strains of *Xylella fastidiosa*

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ABSTRACT

Virulence of *Xylella fastidiosa* strains to grapevine varies from avirulent to highly virulent. Weakly virulent strains multiply and move systemically in the plant, but produce only minor symptoms in the host. In the greenhouse, weakly virulent strains of *X. fastidiosa* were used to inoculate two of the lower internodes of rooted cuttings of *Vitis vinifera* 'Carignane' with 10^5-10^6 bacteria per inoculation site. Inoculation of these plants 2 weeks later with a highly pathogenic strain of the bacterium resulted in a lower incidence and severity rating for Pierce's disease than occurred in plants that had not been protected with weakly virulent strains. Avirulent strains were ineffective in these cross protection tests. Inoculation with the weakly virulent strain needed to precede the challenge inoculation by 1-2 weeks, or more, to be effective.

INTRODUCTION

Xylella fastidiosa strains cause economically important diseases in hosts such as almond, grapevine, peach, plum, and urban shade trees (HOPKINS, 1977). Pierce's disease (PD) is primarily responsible for the failure of bunch grapes (*Vitis vinifera* L. and *V. labrusca* L.) in southeastern USA (HOPKINS *et al.*,1974). Isolates of *X. fastidiosa* from grapevine and other hosts vary in virulence from avirulent to highly virulent (HOPKINS, 1985). Avirulent isolates are able to colonize grapevine but not systemically. Weakly virulent isolates colonize systemically but more slowly than highly virulent isolates.

There are numerous examples of resistance being induced in a susceptible host by prior inoculation with a nonpathogenic, or pathogenic, isolate of a fungus, bacteria, or virus (KUC, 1990, LOBENSTEIN, 1972). *X. fastidiosa* causes vascular wilt-type diseases, and induced resistance is known to occur with the Fusarium vascular wilt pathogen (BILES & MARTYN, 1989). The purpose of this study was to determine whether induced resistance could be demonstrated for PD of grapevine.

MATERIALS AND METHODS

Isolates of *X. fastidiosa* evaluated as the inducing agent included: PD-1, a grapevine isolate that became weakly virulent during serial transfer; PD-4, a grapevine isolate that became avirulent during serial transfer; 83G-1, a goldenrod isolate that is avirulent to grapevine; and Syc86-1, a sycamore isolate that is weakly virulent to grapevine. Challenge inoculations were with the highly pathogenic isolates, PD88-5A and PD89-1, obtained from grapevine with PD symptoms in 1988 and 1989, respectively. Rooted green cuttings of *V. vinifera* 'Carignane' with a minimum of 8 nodes at first inoculation were used in these tests.

For inoculum, *X. fastidiosa* strains were grown on PD3 medium at 28C for 4-6 days, 10-12 days with strain 83G-1. Bacterial suspensions in a phosphate buffer (pH 7.0) were adjusted turbidimetrically to 10^7-10^8 colony-forming units per ml. The avirulent or weakly virulent isolates were used to inoculate two of the lower internodes of the grapevine by placing one drop (0.05 ml) of inoculum on the internode and piercing the internode 3-5 times through the drop, which resulted in the inoculum being pulled into the plant. Approximately 5 x 10^5 to 5 x 10^6 bacteria were inoculated into each internode. The challenge inoculation was usually done 2 weeks later into a single internode, using the methods described for the inducing strain. Inoculated plants were maintained in the greenhouse at 28-33C in the daytime and 20-25C at night.

Disease incidence, based on symptoms and confirmed by culturing, was recorded every 2 weeks. Four months after the challenge inoculation, severity ratings of PD symptoms were made using an arbitrary 0-5 scale: 0 = no symptoms, 1 = leaf marginal necrosis (MN) in the basal leaf, 2 = MN in one-third

or fewer of the leaves, 3 = MN in 30-50% of the leaves, 4 = MN in 50-75% of the leaves, and 5 = MN in all of the leaves or a dead plant.

RESULTS

'Carignane' grapevines inoculated with the weakly virulent *X. fastidiosa* isolates, PD-1 and Syc86-1, and challenged 2 weeks later with a highly virulent isolate either failed to develop Pierce's disease symptoms or developed milder symptoms than developed on buffer controls challenged 2 weeks later with the virulent isolate (Table 1). Grapevines inoculated with the avirulent isolates, 83G-1 and PD-4, and challenged 2 weeks later with the highly virulent isolate developed symptoms similar to the controls. The weakly virulent isolates sometimes produced visible symptoms as seen in the PD-1/Buffer treatment.

Table 1. Induced resistance to Pierce's disease (PD) of grapevine by avirulent or weakly virulent isolates of *Xylella fastidiosa*.

Induction/challenge treatment[y]	Incidence of PD (%)	Disease severity (0-5)[z]
Buffer/PD89-1	50	2.5
PD-1/Buffer	50	1.0
PD-1/PD89-1	67	1.3
83G-1/PD89-1	50	2.25
Syc86-1/PD89-1	0	0
PD-4/PD89-1	50	2.5

[y]Induction inoculation was into the third and fifth internodes from the base of the plant and the challenge inoculation was into the fourth internode 2 weeks later.

[z]Severity ratings of PD symptoms were made on an arbitrary scale of 0-5 with 0 = no symptoms, 3 = marginal necrosis in 30-50% of the leaves, and 5 = marginal necrosis in all leaves, or a dead plant.

With the weakly virulent isolates as the inducing agent, the induction inoculation needed to precede the challenge inoculation by 1 to 2 weeks, or more, to be consistently effective (Table 2). Induced resistance was generally not effective when the induction and challenge inoculations were done on the same day. The disease reaction observed with a 1 week interval was variable. In some cases, induced resistance was expressed both as reduced incidence of disease and reduced symptom severity, but in others only a reduction in symptom severity was observed.

Table 2. Effect of the interval between induction inoculation and challenge inoculation on induced resistance to Pierce's disease (PD).

Induction/challenge treatment[y]	Inoculation interval(wks)	Incidence of PD(%)	Disease severity(0-5)[z]
PD-1/PD89-1	0	100	4.0
Syc86-1/PD88-5A	0	100	3.0
PD-1/PD89-1	1	100	3.7
Syc86-1/PD88-5A	1	67	1.3
Buffer/PD89-1	2	67	3.3
Buffer/PD88-5A	2	100	4.0
PD-1/PD89-1	2	33	1.0
Syc86-1/PD88-5A	2	100	1.3
PD-1/PD89-1	4	67	1.3
Syc86-1/PD88-5A	4	100	3.7

[y]The third and fifth internodes from the base of the plant were inoculated with the induction isolate and the challenge inoculation was into the fourth internode.

[z]Severity ratings of PD symptoms were made on an arbitrary scale of 0-5 with 0 = no symptoms, 3 = marginal necrosis in 30-50% of the leaves, and 5 = marginal necrosis in all leaves, or a dead plant.

DISCUSSION

Weakly virulent isolates of *X. fastidiosa* were able to induce resistance, or cross-protection, to Pierce's disease in *V. vinifera* 'Carignane'. These weakly virulent isolates have been shown to multiply and systemically colonize grapevine (HOPKINS, 1985). Systemic colonization may be necessary for induced resistance to occur, since avirulent isolates that are localized in the plant did not provide protection against virulent isolates. The induction period appeared to be 1-2 weeks, challenge inoculation at 0 or 1 week usually resulted in severe disease symptoms.

Pierce's disease prevents the growing of *V. vinifera* in the southeastern USA (HOPKINS *et al.*, 1974). Perhaps, induced resistance by mild strains of the Pierce's disease bacterium could provide a means of growing bunch grape cultivars that previously would not grow in Florida. However, these experiments were conducted on rooted cuttings in the greenhouse and were only 6 months in duration. We do not know whether the induced resistance will work in the vineyard. If it provides protection in the vineyard, will the mild strains continue to colonize the grapevine and provide protection for more than one season? Field experiments currently are being conducted.

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Evidence that restricted bacterial spread in xylem is involved in the bacterial wilt resistance of tomato

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Abstract. The tomato wilt caused by race 1 of the soil-borne bacterium *Pseudomonas solanacearum* is a vascular disease which severely affects crops in tropical and subtropical lowlands. The bacteria invade vascular tissues from artificial or natural openings like the emergence of secondary roots. Resistant cultivars are widely used to control the disease, although this strategy is limited by the large variability of the strains and host genotype-environment interactions. Tomato resistance mechanisms do not result from absence of root penetration by the bacteria because latent infections were observed in all symptomless resistant plants tested. Tomato grafting experimentations proved that resistance results from the restriction of bacterial spread: susceptible scions did not wilt when grafted on resistant root-stocks and bacterial spread was similar to resistant scions grafted on resistant root-stocks. In contrast, resistant scions grafted on susceptible root-stocks were heavily colonized resulting in typical wilt. A study of the density and spatial distribution of bacteria in the stem of 10 cultivars (from USA, Taiwan, French West Indies) with different levels of resistance indicated that all wilted plants were similarly colonized regardless of the cultivar. Resistance to wilt was found to be negatively correlated with the detection frequency of *P. solanacearum* at mid-stem. These results provide first evidences that the limitation of the spread of bacteria into the xylem is required for tomato wilt resistance involving constitutive or induced defence mechanisms.
Keywords : *Pseudomonas solanaceaum*, tomato, latent infections, wilt resistance, xylem colonization.

Bacterial wilt (BW) caused by *Pseudomonas solanacearum* (Smith) Smith (1896) is one of the most damaging tropical and subtropical disease of a wide range of crops, especially vegetable (BUDDENHAGEN, 1986; HAYWARD, 1991). This soilborne bacterium causes a vascular infection which makes very difficult to control the disease with chemicals and to apply sanitation measures. Selection for

disease resistance remains the best strategy even though properties of resistant cultivars may fluctuate from breeding to cropping areas, due to extreme variability and adaptation of the pathogen (PERSLEY, 1986).

There is an evident need of complementary research to characterize BW resistance mechanism(s) developed by different hosts for improving selection strategies. In potato, latent infection of tubers by *P. solanacearum* was reported by CIAMPI *et al.* (1980) from symptomless plants. Then, BOWMAN & SEQUEIRA (1982) indicated that BW resistance in potato was associated with limited bacterial invasiveness, and tolerance to high bacterial density. In tomato, latent infections were recently reported from symptomless BW resistant tomato stems (PRIOR *et al.*, 1990a). Latent infections were observed both in cultivars and sources of resistance 'Hawaï 7996' and 'CRA 66'. This demonstrates that resistance does not arise from physical barriers to *P. solanacearum* root penetration but from tolerance of vascular tissues to high bacterial populations. In addition, stem colonization by the bacterium was considered faster in susceptible cultivars compared to resistant ones. This work was undertaken to evaluate the relation between tomato BW resistance and bacterial invasiveness.

Materials and methods

Plant production and inoculation. Seeds of tomato cultivars that differed in resistance to BW (Table I) were settled into steam disinfested organic soil. Ten-day-old seedlings were transplanted in individual pots placed in an insect-proof greenhouse. *P. solanacearum* strain GMI 8217 is a fluidal, spontaneous mutant resistant to 200 µg/ml streptomycin and 50 µg/ml rifampicin which was selected from wild-strain GT1 (encoded CFBP 3256) previously described (PRIOR & STEVA, 1990; PRIOR *et al.*, 1990b). Virulent, fluidal wild-type colonies were selected after 48 hr growth at 30 C on Kelman's tetrazolium chloride medium (TZC) (KELMAN, 1954) with antibiotics (selective medium) and suspended in sterile distilled water (SDW). These colonies were restreaked on the same medium without TZC. After 48 hr growth on selective medium free of TZC, cells were harvested by flooding the plates with SDW, and inoculum consisted in suspension of 2.10^7 cells/ml adjusted spectophotometrically (10^7 cells/ml correspond to $A_{(650\ nm)}$= 0,01). Two milliliter of inoculum were poured around the base of plants and a scalpel was inserted 4-5 cm into the soil to cut the roots along one side. From three days after inoculation, symptom development was recorded every other days up to 34 days.

Table I Origin and principal characteristics of selected tomato cultivars.

Cultivar	Origin	Country	Growth (1)	Susceptibility (2)
Floradel	Petoseed	United-States	u	S
Caraïbo	INRA	Antillies	d	R
PT 4165	AVRDC	Taïwan	d	MR
Caracoli	INRA	Antillies	u	MR
CRA 66	INRA	Antillies	u	R
FMTT 3	AVRDC	Taïwan	d	R
CRA 90-30	INRA	Antillies	d	R
Hawaï 7996	Univ.	United-States	d	R
CLN 657	AVRDC	Taïwan	u	R
Calinago	INRA	Antillies	d	R

(1) : Determinated (d) or undeterminated (u) growth. (2) : The cultivar was indicated susceptible (S), moderatly resistant (MR) or resistant (R) to bacterial wilt.

Table II Comparison of *P. solanacearum* spread into plants resulting from different combinations of cleft graftings between susceptible (Floradel) and résistant (Caraïbo) tomato genotypes .

	log cfu/g FM (1)				% colonization			
Graft (2)	Collar	< GS (3)	> GS	10cm>GS	Collar	< GS	> GS	10cm>GS
Flo/ CBO	5,02 ^{a}	3,94 ^{a}	3,53 ^{a}	3,56 ^{a}	95,5	68,5	39,3	20,2
CBO/CBO	5,22 ^{a}	4,42 ^{a}	4,09 ^{ab}	2,61 ^{a}	100	64,3	35,7	28,6
Flo/Flo	7,68 ^{b}	7,55 ^{b}	6,88 ^{c}	6,08 ^{b}	100	100	93,8	87,5
CBO/Flo	7,45 ^{b}	7,12 ^{b}	5,78 ^{cb}	4,92 ^{c}	100	100	91,7	83,3

(1) Spiral counts were expressed as log cfu per gram fresh matter. Data with different superscripts are significantly different according to variance analysis (P<0,05). (2) Scion / root-stock; Flo = floradel and Car = caraïbo. (3) Spiral counts below <, above > and 10 cm above graft-scar (GS).

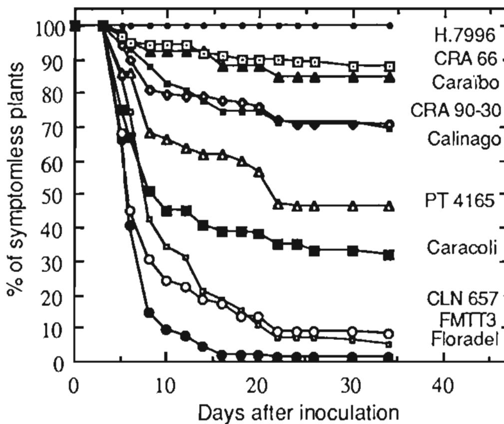

Figure 1 Field resistance of the selected cultivars after inoculation with *P. solanacearum.*

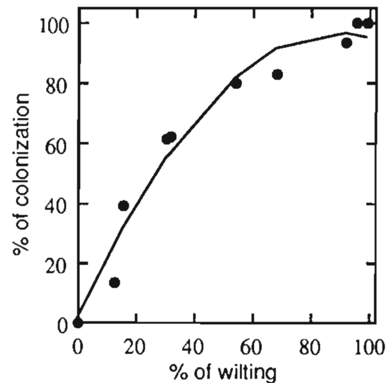

Figure 2 Correlation between bacterial spread and wilt frequencies within cultivars (•).

Cleft grafting. Two tomato cultivars were selected for susceptibility and resistance to BW: Floradel (Flo) and Caraïbo (Car), respectively. Grafting was performed with 4 wk-old root-stocks and 3 wk-old scions. Root-stocks were topped above the second leave and foliar surface of scion was reduced by cutting leaves by half. Grafts were holded using a parafilm sheet and plants were protected under a shadehouse (8-10 days) until shoot was well established. Scion/root-stock grafting experimentation consisted in 110 Flo/Car, 40 Car/Flo, 20 Flo/Flo and 20 Car/Car. Plants with homogeneous size were inoculated 10 days after grafting. Alcohol disinfected stem cuttings (2 cm) were sampled 3 weeks after inoculation at collar, below and above graft scar and 10 cm above graft scar.

Field trial. For all cultivars, 100 plants with 3-4 fully expanded leaves were infected and planted next day in the field. Statistical device consisted in five randomized complete blocks, each of them containing 6 m planting rows of all cultivars at the rate of 20 plants. Ten wilted plants per cultivar were sampled and a 2 cm lengh fragment was cut off at midstem. Symptomless plants were collected 34 days after inoculation, with a maximum of 35 plants per cultivar and fragments were sampled at taproot, collar and midstem.

Bacterial counts. Taproots were crushed and other cuttings were placed in 4 ml SDW and stored at 10 C for 15 hr to allow deposition of bacteria. Extracts were streaked on selective medium using a Spiral plater and colonies were counted after incubation of plates for 48 hr at 30 C. Technical lower limit for Spiral counts was 10^2 cfu/ml. Populations of *P. solanacearum* counted in tomato extracts were expressed as bacteria cfu/g of fresh matter (cfu/g FM) and were log-transformed. A variance analysis test was used to compare data.

Results

Bacterial wilt. Typical BW symptoms and death were observed on 50% Flo/Flo and 25% Car/Flo plants, whereas no diseased plants were noted within Flo/Car and Car/Car. The first symptoms in the field were observed 5 days after inoculation. For all cultivars, the disease progression curves were expressed as the rate of symptomless plants (Figure 1) which decreased rapidly from day 3 to 10, depending on the susceptibility to BW. Twenty days after inoculation there was no more significant evolution of the disease. Different level of wilt were observed ranking from highly susceptible 'Floradel' (no surviving plant) to highly resistant 'Hawaï 7996' (100% symptomless plants).

Bacterial spread. Spatial colonization and GMI 8217 density in conducing vessels of the different combinations of resistant and susceptible root-stocks and scions are presented Table II. Colonization rates at collar were not significantly different between combinations. There were no differences in *P. solanacearum* spread and densities at upper level of plants grafted with the same root-stock, but colonization rates (80-90% positive counts) and densities were significantly higher (almost 2 to 3 log units) into plants with susceptible stock compared to resistant ones (20-40% positive counts). Regardless the cultivar, all the plants placed in the field were colonized by GMI 8217 at the collar (75-100% positive counts). No difference was observed between the bacterial counts from wilted plants ($3,8.10^9 \pm 0,4\ 10^9$ cfu/g MF average). In addition, the average number of GMI 8217 counts (log) at collar ($6,88 \pm 0,8$) was greater than at mid-stem ($4,13 \pm 0,8$)

The 10 cultivars statistically differed in colonization rate at mid-stem, the most resistant to BW being the less colonized. To compare all the cultivars, the bacterial colonization was expressed with consideration to BW frequencies according to the formula: %wilted plants (100% colonization) + [%symptomless plants x %colonization]. A strong negative correlation ($y = -0,012\ x^2+2.14x+1.2;\ r^2=0.97$) appeared between the pondered bacterial colonization and BW frequencies (Figure 2).

Discussion

All cultivars resistant and moderatly resistant to BW tested, except FMTT 3 and CLN 657 from Taïwan (AVRDC), were harboring the expected level of resistance in Guadeloupe environment. Resistance was overcome for FMTT 3 and CLN 657 but not for PT 4165, although it was selected in the same area. Such variation in the performance of resistance cannot be attributed solely to variation in pathogenicity of strains, but is consistent with the concept that interaction between host genotype and environment is prevalent to score with resistance.

Bacterial densities reported from all wilted plants were similar. This indicated that resistance of a cultivar to BW was not reliant on a threshold for *P. solanacearum* density. All plants were colonized regardless the origin of the resistant cultivar, suggesting that latent infection is a general phenomenon. While there were no significant differences between resistant cultivars for the bacterial counts at mid-stem, the level of resistance for a cultivar was found to be negatively correlated with the detection frequencies at this level. Further evidence is given by grafting susceptible scions on resistant root-stocks in which the properties of the

resistant root-stock was confered to the scion by the limitation of bacterial invasiveness. These results provide the first evidence that the capability for the tomato to limit the spatial colonization by *P. solanacearum* is required for BW resistance. This is consistent with earlier reports on resistance to other vascular diseases (ELGERSMA *et al., 1972* ; BECKMAN *et al.*, 1972). Numerous hypothesis, involving constitutive or induced defence mechanisms may explain restriction of bacteria in resistant plant compared to susceptible: differences in vascular anatomy, gum and tylose production, nature of plant-extract, bacterial growth and production of exopolysaccharides. It is very likely that latent infection rate at mid-stem will provide breeders with a new criterion, complementary to wilt frequency. This new descriptor for tomato BW resistance will be useful to select rapidly for more stable resistance, by understanding influences of factors like temperature, light, water stress or nematodes on breakdown in field resistance of cultivars.

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Advances in biological control to tomato bacterial wilt using genetically engineered avirulent mutants of *Pseudomonas solanacearum*

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Abstract : Selection of resistant cultivars is the main strategy used to control bacterial wilt of tomato. Recent advances in knowledge of the genetic determinants of *Pseudomonas solanacearum* pathogenicity suggested that a biological control method could be developed using genetically engineered avirulent mutants of *P. solanacearum*. Three virulent strains (GA2, GA4, and GT4) collected in Guadeloupe (French West Indies) were transformed to avirulence by insertion of an Ω-Km interposon within the *P. solanacearum hrp* gene cluster. The resulting mutants (GMI 8171, GMI 8172, and GMI 8173, respectively) were tested for the ability to control bacterial wilt in challenge inoculation experiments conducted under greenhouse conditions in Guadeloupe.

Upon root inoculation of the susceptible tomato cv. Floradel, the mutants colonized taproot and collar tissues, but did not reach the fruits. Moreover, presence of the avirulent bacteria did not affect fruit production. Inoculation of cv. Floradel with avirulent mutants was correlated with a reduction in disease severity when the plants were subsequently inoculated with the virulent strain, GMI 8217. Despite the reduction in disease severity, virulent bacteria were still able to multiply in protected tissue, attaining population levels five to six order of magnitude greater than that attained by the co-resident, avirulent bacteria.

Based on these results we conclude that protection is unlikely to result from general exclusion of the virulent strain.

Keywords : *Pseudomonas solanacearum*, tomato, bacterial wilt, biological control, avirulent mutants, bacteriocins.

INTRODUCTION

Bacterial wilt caused by *Pseudomonas solanacearum* is a serious disease on several agriculturally important plants in tropical, subtropical and warm temperate regions of the world (HAYWARD,1991). There have been several attempts to control bacterial wilt using antagonistic bacteria (KEMPE & SEQUEIRA, 1983; ANURATHA & GNANAMANICKAM, 1990) or spontaneous avirulent mutants of *P. solanacearum* (Mc LAUGHLIN *et al.*, 1990; TANAKA *et al.*, 1990), but these have met with limited success.

We began to investigate the use of genetically-defined, avirulent mutants of *P. solanacearum* for biological control. For this purpose we have employed Tn5-induced *hrp* mutants (BOUCHER *et al.*, 1985) that retain the ability to colonize tomato plants without causing disease (TRIGALET & DEMERY, 1986). Preinoculation of tomato plants with these avirulent mutants has been correlated with decreased disease severity upon challenge inoculation with a wild type, virulent strain (TRIGALET & TRIGALET-DEMERY, 1990).

Recently, we have constructed a new series of avirulent mutants by inserting the Ω-Km interposon adjacent to the site of one of the original Tn5 insertions (MARIE, 1989). Three such mutants, each derived from a different virulent strain of *P. solanacearum*, were examined for the ability to control bacterial wilt and for their effect on fruit yield. The experiment was conducted in a contained environment on the island of Guadeloupe (French West Indies), where bacterial wilt is endemic.

MATERIALS AND METHODS

Construction of avirulent mutants :

We have previously determined that, of the original Tn5-induced *hrp* mutants of *P. solanacearum* (BOUCHER *et al.*,1985), strain GMI 1353 showed greatest potential as a biocontrol agent (TRIGALET & TRIGALET-DEMERY, 1990). Therefore, using GMI 1353 as the prototype strain, we constructed a new series of *hrp* mutants by insertion of the Ω-Km interposon (FELLAY *et al.*, 1987) at an EcoRV site near to the Tn5 insertion site in GMI 1353 (MARIE, 1989; table I). Like GMI 1353, these Ω-Km mutants were avirulent on tomato plants and still able to multiply within the host plant. We used the Ω-Km interposon in order to avoid transposition-associated reversion which is potentially a problem with Tn5-induced mutations.

Inoculum :

Bacteria were grown at 30°C on peptone medium (bacto-peptone 10 g, glucose 5 g, yeast extract 1 g, casamino-acids 1 g, distilled water 1 l; solid medium supplemented with bacto-agar 15 g, triphenyl-tetrazolium chloride 50 mg, and the

following antibiotics : kanamycin (Km) 50 mg, streptomycin (Sm) 200 mg, rifampicin (Rif) 50 mg). Inoculum was prepared from 24 hrs broth cultures by adjusting the bacterial density with distilled water (A_{650} = 0.01 corresponding to 10^7 CFU . ml^{-1}).

Table I : characteristics of the bacterial strains :

code number	characteristics	reference or source
Virulent strains:		
GA2	wild type (eggplant, Guadeloupe, 1986)	PRIOR &STEVA,1990
GA4	wild type (eggplant, Guadeloupe, 1983)	PRIOR &STEVA,1990
GT4	wild type (tomato, Guadeloupe, 1984)	PRIOR &STEVA,1990
GT1	wild type (tomato, Guadeloupe, 1985)	PRIOR &STEVA,1990
GMI 8217	spontaneous Sm^R Rif^R derivative of GT1	A. TRIGALET
Avirulent strains :		
GMI 8171	Ω-Km induced derivative of GA2	MARIE,1989
GMI 8172	Ω-Km induced derivative of GA4	MARIE,1989
GMI 8173	Ω-Km induced derivative of GT4	MARIE,1989

Plant material and root inoculation :

The experiments were conducted in a contained environment in Guadeloupe during two separate seasons (cool season : November-April, 20/27°C night/day ; warm season : May-October, 23/29°C). Susceptible tomato plants (cv. Floradel) were grown in 7x7 cm pots containing steam-disinfected greenhouse soil. For root inoculation with the avirulent mutants, 3 to 4 week old plants were soaked in a bacterial suspension (2 x10^9 CFU.ml^{-1}) for 5 min. Four weeks later, the plants were challenge inoculated with the virulent strain GMI 8217 (3 ml per plant of an inoculum of about 10^7 CFU. ml^{-1} was poured onto the wounded roots).

Bacterial isolation tests :

To monitor bacterial populations *in planta*, 2 cm stem segments were surface-sterilized and placed in 5 ml sterile distilled water at 10°C for 12 hrs. Bacteria which streamed from the xylem vessels were counted following dilution plating on selective medium. Bacterial densities obtained with this method were not significantly different from those obtained after crushing the stem segments.

Symptoms and yield notations :

Bacterial wilt symptoms were assessed according to the disease index (DI) scale of WINSTEAD & KELMAN (1952). The control rate is expressed as the percentage of protection :

$$\%P = 1 - \frac{\text{average DI of the avirulent/virulent combination}}{\text{average DI of the virulent control}} \times 100$$

Parameters of yield (number of flowers per plant, number of fruits per plant, mean

weight per fruit, yield per plant) were recorded during cool and warm seasons on plants inoculated with avirulent mutants GMI 8171 and GMI 8172, respectively.

Bacteriocin production :

Direct antagonism between avirulent and virulent strains was studied. 10 µl of a bacterial suspension or filtrate (Φ = 0.22 µm) of a producer strain was spotted on a bacterial lawn of an indicator strain. After incubation at 30°C for 24 hrs, the diameter of inhibition zone was measured.

RESULTS

Colonization of tomato plants by avirulent strain GMI 8172 :

Three months after root inoculation with strain GMI 8172, corresponding to the end of the fruit production period, bacteria were isolated at six points within the plant (taproot, collar, mid-stem, 1st, 2nd and 3rd peduncle). Avirulent bacteria were detected only in taproot (8.51 log CFU/g dry weight) and collar tissues (5.66 log CFU/ g DW).They were never detected in the mid-stem tissue, nor in peduncle tissue. This contrasts with the virulent bacterium, which spreads systemically throughout the diseased plant.

Colonization of tomato plants after inoculation with avirulent strains GMI 8171, 8172 or 8173 and challenge inoculation with virulent strain GMI 8217 :

The three avirulent mutants colonized the collar tissue at much lower densities than the virulent strain (table II). Nevertheless, preinoculation with the avirulent strains GMI 8171 or GMI 8172 was correlated with a significant reduction in colonization by the virulent strain GMI 8217. This was not the case with avirulent strain GMI 8173, consistent with the lower protection rate obtained.

Table II : bacterial densities at the collar level and control rate of each combination :

Avir. / Vir. combination	Avirulent strain		Virulent strain		Control rate	
	Col. pl. (%)*	log CFU/gDW**	Col. pl. (%)	log CFU/gDW	DI	% P
8171 / 8217	75.5	4.64 a	100	9.93 a	0.87	54.1
control 8217	-	-	100	10.83 c	1.89	
8172 / 8217	83.0	5.42 a	98.1	10.17 ab	0.96	61.1
control 8217	-	-	100	10.89 c	2.46	
8173 / 8217	82.7	5.15 a	98.2	10.55 abc	2.05	31.9
control 8217	-	-	100	10.74 bc	3.02	

Mean of 56 plants for each combination. In each column, values followed by the same letter are not significantly different at the level of 5 %.* percentage of colonized plants.** DW = dry weight.

Incidence on fruit production :

Experiments performed during cool and warm seasons showed that the parameters of yield of tomato plants inoculated with avirulent mutants GMI 8171 or 8172 were not significantly different from those of non-inoculated plants.

Bacteriocin production :

We showed that the virulent strain GMI 8217 produces, *in vitro,* a bacteriocin active against the three avirulent mutants and against numerous wild type strains of *P. solanacearum* collected in Guadeloupe. This bacteriocin is also produced *in planta* in tomato stems infected with strain GMI 8217.

CONCLUSIONS AND PERSPECTIVES

Tomato plants that were inoculated with Ω-Km-induced avirulent mutants exhibited reduced disease severity upon subsequent challenge inoculation with virulent *P. solanacearum*. Importantly, inoculation with the avirulent mutants did not have a detrimental effect on fruit yield. In the case of the avirulent mutants GMI 8171 and GMI 8172, protection was correlated with a significant reduction in multiplication of the virulent strain GMI 8217. However, in collar tissue from co-inoculated plants, avirulent mutants were consistently 5 or 6 orders of magnitude less abundant than the virulent strain. The relative overabundance of virulent bacteria in double-inoculated tissue makes direct antagonism and competition for available resources unlikely mechanisms for control of disease ; thus, we consider that protection could result from the induction of host resistance mechanisms.

We have determined that the virulent strain GMI 8217 produces a bacteriocin that is active against numerous strains of *P. solanacearum*, including the three avirulent mutants used in these experiments. This result may explain the low protection rates we obtained. As avirulent bacteriocin-producing strains of *P. solanacearum* are potential biocontrol agents against bacterial wilt (CHEN & ECHANDI, 1984 ; HARA & ONO, 1991), it may be informative to test in future experiments avirulent *hrp* mutants that have bacteriocin-resistant and/or bacteriocin-producing phenotypes.

For several reasons (including the influence of the contained environment, distribution and concentration of applied inoculum, abiotic factors) these experiments did not reproduce the conditions under which bacterial wilt occurs in nature. Therefore, to further examine the efficacy of these *hrp* mutants as biological control agents, additional tests must be conducted under field conditions, where infection by the pathogen occurs naturally.

Résumé: La création de variétés résistantes est le principal moyen de lutte contre le flétrissement bactérien des Solanacées. Les progrès récents sur les déterminants génétiques du pouvoir pathogène de *Pseudomonas solanacearum* nous ont permis d'envisager une stratégie de lutte biologique utilisant des mutants avirulents de *P. solanacearum* obtenus par génie génétique. Trois souches sauvages isolées en Guadeloupe (GA2, GA4 et GT4) ont été transformées par insertion d'une cassette Ω-Km dans un site EcoRV de la région *hrp*. Les mutants avirulents obtenus, GMI 8171, 8172 et 8173 respectivement, ont été testés en serre en Guadeloupe. Après inoculation au niveau des racines, ces mutants colonisent le pivot et le collet de plants de tomate sensibles (cv. Floradel), mais n'atteignent pas les fruits. De plus, la présence d'un mutant avirulent dans la plante n'affecte pas la production de fruits. Les trois mutants ont un effet protecteur vis-à-vis d'une surinfection des plantes avec une souche virulente (GMI 8217). Au niveau du collet, la densité des mutants avirulents est nettement inférieure à celle de la souche virulente, ce qui suggère que la protection ne résulte pas d'un phénomène d'exclusion générale. Par ailleurs, nous avons montré que la souche GMI 8217 produit *in vitro* et *in planta* une bactériocine active contre de nombreuses souches de *P. solanacearum*, dont les trois mutants avirulents. Au vu de ces résultats, nous pouvons émettre l'hypothèse d'un mécanisme de résistance induite de l'hôte.

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Characteristics of sugarcane resistance to leaf scald disease caused by *Xanthomonas albilineans*

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ABSTRACT. A spontaneous mutant of *Xanthomonas albilineans*, resistant to streptomycin and rifampicin, was used to study the bacterial colonization in sugarcane and the symptom expression of leaf scald disease (LSD). Twelve sugarcane clones with different resistance levels to LSD were inoculated by the decapitation technique. The disease severity and the bacterial populations were recorded three months after inoculation. The populations of *X. albilineans* were measured by isolation on a selective medium in three different sugarcane tissues: the apex, a single node section in the upper third and a single node section in the lower third of the stalk. Disease severity varied between 0% for the most resistant and 99.5% for the most susceptible clone. All the clones were colonized by the pathogen but the greatest densities were found in the susceptible ones. More bacteria were generally detected in the upper part than in the lower part of the stalk. There was a high correlation between disease severity and pathogen populations in sugarcane. Best differentiation of clones was obtained by analysis of bacterial concentrations in the upper third section. In the view of the results obtained, screening procedures for LSD can be based on *X. albilineans* population densities in sugarcane.

KEYWORDS: sugarcane, *Xanthomonas albilineans*, leaf scald, resistance, bacterial populations.

Leaf scald, a vascular disease caused by *Xanthomonas albilineans* (Ashby) Dowson, is one of the major diseases of sugarcane (RICAUD & RYAN, 1989). It occurs in most sugarcane producing countries of the world and recently increased at alarming rates in different areas such as in Dominican Republic, Florida, Guadeloupe and Mauritius. The reasons of these recent outbreaks are not yet known. Leaf scald disease (LSD) can cause severe yield losses. In Guadeloupe, it has been shown to cause, in susceptible cultivars, great reduction in yield in addition to reduced sugar content and juice purity (P. ROTT, unpublished results).

One of the troublesome aspects of leaf scald is that many sugarcane clones can tolerate the pathogen without exhibiting any symptom at all, or the symptoms are so inconspicuous as to escape detection (ROTT *et al.*, 1988a). Planting healthy seedcane and using resistant cultivars are considered to be the most efficient means of control. Screening trials for resistance are carried out in many countries where the disease is a problem but assessment of cultivar reaction takes a long time and is difficult. They are generally based on artificial inoculation of sugarcane and observation of symptoms (KOIKE, 1972). This procedure can be erratic especially when symptoms do not develop. No information is avalaible at present about the colonization level of *X. albilineans* in sugarcane tissues. The objective of our study was to examine quantitatively the disease and the bacterial populations in sugarcane clones differing in resistance to LSD.

MATERIALS AND METHODS. *Bacterial strain*. A spontaneous mutant of *X. albilineans*, resistant to 50 ppm streptomycin and 50 ppm rifampicin, was used to inoculate sugarcane. *Sugarcane clones*. Single eye cuttings of twelve sugarcane clones were soaked for 48 hours in water at ambient temperature and then hot water treated at 50 C for three hours to eliminate natural infections by *X. albilineans*. They were grown in a greenhouse for four months and then planted in the field: 8 to 26 stools per clone without replication.

The eight months old stools were divided into two equal groups and their stalks either inoculated with the pathogen or with water (control). *Inoculation technique*. Inoculation was done by the decapitation technique i.e. stalks were cut above the growing point (third dewlap from the top) with pruning shears that were previously dipped in a bacterial suspension (10^8 colony forming units or cfu/ml). The same inoculum was also sprayed on the section of the stalk just cut. *Sampling procedure*. Twenty five stalks per clone were randomly sampled three months after inoculation and pooled in groups of five stalks. The symptoms were recorded before removal of the different tissues to be examined: the apex (approx. 1 cm x 2 cm), a 5 cm single node section in the upper third and a 5 cm single node section in the lower third of the stalk. An internode section was also taken in the mid third of each stalk to determine if the cane was infected. *Disease assessment*. Symptoms of LSD were recorded for each stalk and disease severity was calculated as follows: DS = [(1FL + 2ML + 3CW + 4N + 5D) / 5T] x 100; FL = number of stalks with few pencil-line streaks (one or two), ML = number of stalks with many pencil-line streaks (more than two per leaf), CW = number of stalks with leaf chlorosis or whitening, N = number of stalks with leaf necrosis, D = number of dead stalks or stalks with side-shooting, T = total number of inoculated stalks. *Measurement of infected stalks*. A freshly-cut surface of each internode section was blotted in duplicate onto the selective medium which consisted of Wilbrink's modified medium (ROTT *et al.*, 1988b) amended with 50 mg/l cycloheximide, 12.5 mg/l benomyl, 50 mg/l streptomycin and 50 mg/l rifampicin. Growth of *X. albilineans* was recorded after a 7-day incubation at 28 C. *Measurement of population densities*. The apices and node sections were weighted and then homogenized in 200 to 400 ml of TBS buffer (Tris 2.42 g, NaCl 8 g, distilled water 1000 ml, pH 7.5) with a blender. Serial dilutions of the extracts were plated in triplicate on the selective medium. Bacterial colonies were counted after a 7-day incubation at 28 C. *Statistical analysis*. Computer programs of Stat-Itcf 4.0 (ITCF

Table 1. Disease severity and bacterial populations three months after inoculation of twelve sugarcane clones with *X. albilineans* GPE 5 SR.

Clone (resistance level[w])	Disease severity	Log_{10} [(cfu/g fresh weight)+1] Lower third node	Upper third node	Apex
CP 68 1026 (HS)	99.5[x] *(55.9)*a	7.97 *(57.2)*a	8.72 *(57.0)*a	8.94 *(57.4)*a
Q 63 (HS)	94.5 *(47.6)*ab	7.14 *(52.2)*ab	8.12 *(52.9)*a	8.13 *(46.6)*b
B 69 379 (S)	61.0 *(38.4)*bc	5.64 *(29.0)*cde	7.45 *(46.3)*b	7.91 *(44.5)*b
B 64 277 (S)	93.4 *(52.9)*a	6.52 *(44.6)*abc	7.28 *(42.2)*bc	7.67 *(39.8)*b
M 147 44 (S)	71.7 *(38.7)*bc	6.37 *(41.5)*bcd	7.08 *(38.0)*c	7.62 *(39.4)*b
B 69 566 (S)	79.0 *(34.6)*c	5.84 *(31.9)*cde	7.06 *(36.6)*c	7.52 *(36.6)*b
CP 68 1067 (HS,I)	35.6 *(17.7)*d	4.21 *(17.2)*ef	6.36 *(26.6)*d	5.79 *(31.7)*bc
R 570 (S,I,R)	53.5 *(29.8)*c	5.47 *(27.0)*de	6.09 *(22.8)*d	6.10 *(23.2)*cd
M 31 45 (S,R)	12.5 *(15.7)*d	4.79 *(19.1)*ef	5.50 *(15.8)*e	1.40 *(13.0)*d
B 80 08 (R)	11.1 *(14.8)*d	5.48 *(29.9)*cde	5.34 *(14.8)*e	ND[y] *(9.0)*d
Co 11 48 (S,R)	3.2 *(13.9)*d	3.03 *(11.7)*f	4.40 *(7.8)*f	1.74 *(12.8)*d
Co 64 15 (R)	0.0 *(6.0)*d	1.48 *(4.7)*f	4.33 *(5.2)*f	1.22 *(12.0)*d
RSE[z]	*(6.95)*	*(8.85)*	*(4.81)*	*(8.27)*

[w] Data from the bibliography: HS = highly susceptible, S = susceptible, I = intermediate or tolerant and R =resistant.
[x] Values are the means of five replications of five plants each. Values in parentheses correspond to the data transformed to ranks. Means in a column followed by the same letter are not significantly different at P = 0.05 according to the test of Newmann-Keuls.
[y] bacteria not detected.
[z] Residual Standard Error.

Boigneville, 91720 Maisse, France) were used for data analysis. Disease severities and population density estimates were transformed to ranks according to CONOVER (1980) and then examined by analysis of variance.

RESULTS. Three months after inoculation disease severity varied between 0% for CO 64 15, the most resistant clone, and 99.5% for CP 68 1026, the most susceptible clone (Table 1). All sugarcane clones were colonized by *X. albilineans*. The percentage of infected stalks reached 100% for the susceptible clones and was not less than 76% for the resistant ones. Bacterial population densities in the stalk varied between 30.2 and 5.2 x 10^8 colony forming units / g of fresh weight (cfu/gFW) in the node sections and between undetectable levels and 8.7 x 10^8 cfu/gFW in the apex. More bacteria were detected in the upper part than in the lower part of the stalk in 11 out of the 12 clones. There was a linear relationship between disease severity and pathogen populations (log base 10 transformation) in the different sugarcane tissues. The correlation coefficients were 0.95, 0.94 and 0.86 respectively for the upper node section, the lower node section and the apex. Regarding the four examined parameters (disease severity and bacterial populations in three sugarcane tissues), best differentiation of clones was obtained by analysis of bacterial concentrations in the upper third section of the stalk and the 12 clones could be easily separated into six groups (Table 1).

DISCUSSION. Resistance of sugarcane to LSD appeared to be inversely correlated with the symptoms severity and the colonization level of vascular tissues by *X. albilineans*. Best differentiation of the sugarcane clones was obtained by measurement of the bacterial population densities in the upper node section of the stalk. In the present study, we established that susceptible clones can be separated from resistant clones by a minimum colonization level of 10^7 cfu/gFW. This level may vary according to several parameters but preliminary studies have shown that *X. albilineans*

populations in the stalk can remain stable for several months even if disease severity evolves (ROTT *et al.*, 1991). Although the basis for clone differences in colonization are not yet known, screening procedures for LSD resistance can be based on pathogen population densities in sugarcane.

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Management of bacterial blight of pea (*Pisum sativum* L.) through chemicals

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Streptocycline @ 100 and 200 µg/ml alone and in combination with 0.2 % Blitox 50 WP (copper oxychloride) when applied as foliar spray at 7 and 15 days intervals significantly reduced the per cent disease index, infection rate, relative area under disease progress curve as compared to control. Seven days spray interval gave significantly better control than the spray applications at 15 days interval. The former treatment also gave highest yield with a net profit of more than Rs.8400/ha. Seed treatment with Streptocycline (250 µg/ml) for 2 and 4 h reduced disease incidence by 95 per cent in the crop raised from artificially inoculated seeds. Combinations of seed treatments with spray application of Streptocycline (100 µg/ml) at 7 and 15 days intervals gave better control than the seed treatment or spray alone. Seed treatment with Streptocycline (250 µg/ml) for 4 h followed by its spray application (100 µg/ml) at 7 days interval gave a maximum disease control of 96 per cent and increased the green pod yield by 70 per cent with a net profit of Rs.16174 and 18406/ha during 1989-90 and 1990-91, respectively.

KEYWORDS: Pea, *Pisum sativum*, Bacterial blight, *Pseudomonas syringae* pv. *syringae*, Management

INTRODUCTION

Bacterial blight (*Pseudomonas syringae* pv. *syringae* van Hall), a seed borne disease of pea is of world wide importance especially in wet temperate regions (TAYLOR & DYE, 1976, WIMALAJEEWA& NANCARROW,1984, JINDAL & BHARDWAJ, 1989). The methods adopted to control the disease include the use of disease free seed (SMITH & CLOSE, 1977, YOUNG & DYE, 1970), chemical seed treatment (WARK,1949, TAYLOR & DYE, 1976, JINDAL & CHOPRA, 1991)

and spray application (JINDAL, 1990). Since these treatments alone have not been able to provide satisfactory control of the disease in the crop raised either from heavily infected seed lot or under too wet conditions, therefore in the present investigation, attempts have been made to formulate the strategy for the management of bacterial blight through combination of seed treatment and spray application of chemicals.

MATERIALS AND METHODS

Field experiments on the management of bacterial blight of pea were conducted during 1988-89, 1989-90 and 1990-91. The experiments were laid out in RBD with three replications and plot size of 4 x 2.5 m^2.The data on disease severity were recorded on 0-5 scale; 0- no diseases, 1- 0.1 to 1.0, 2- 1.1 to 10.0, 3- 10.1 to 25.0,4- 25.1 to 50.0, 5>50 per cent plant area under disease.

To check the secondary spread of the disease Streptocycline, a formulated product of Hindustan Antibiotics Ltd., Pimpri, Poona, containing 90 % streptomycin sulphate and 10 % tetracycline hydrochloride @ 100 and 200 µg/ml alone and in combination with 0.2 % Blitox 50 WP (copper oxychloride) were tested as foliar spray. During 1988-89, the experiment was laid out on the crop raised for seed production inthe experimental area of the University. Before starting spray, 9 plants with various disease levels were tagged ineach plot. First spray was given on March 1,1989 and subsequent 4 and 2 sprays at 7 and 15 days intervals. Data onthe disease progress were recorded before everyspray and relative area under disease progress curve (RAUDPC) was worked out (FRY, 1978). During 1989-90, the trial was conducted by using naturally infected seeds, collected from heavily diseased crop. First spray was given immediately after the appearance of disease symptoms i.e. on January 16, 1990 and subsequent 9 and 5 sprays at 7 and 15 days intervals, respectively. Infection rate of the period of disease progress was also worked out (Van der Plank, 1963). Data on green pod yield were also recorded and net profit for each treatment worked out on the basis of prevailing market prices.

Field trials were also conducted using 2 seed treatments (Streptocycline 250 µg/ml for 2 and 4 h), 2 spray schedules (Streptocycline 100 µg/ml at 7 and 15 days intervals) and their 4 combinations during 1989-90 and 1990-91. Seeds of cv. Lincoln were artificially inoculated by soaking in bacterial cell suspension (1 X 10^8 CFU/ml) for 12 h followed by drying at room temperature. The inoculated seeds were dipped in Streptocycline (250 µg/ml) solution for 2 and 4 h and then air dried at room temperature before sowing. During 1989-90, first spray was given immediately after the disease appearance and subsequent 9 and 5 sprays at 7 and 15 intervals, respectively. During 1990-91 first spray was delayed by 27 days because the primary infection was not apparent until the crop was 70 days old and subsequent 6 and 3 sprays were applied at 7 and 15 days intervals, respectively. The data on the disease incidence were recorded 30 and 70 days after sowing during 1989- 90 and

1990-91, respectively. Final data on the disease were recorded 15 days after the last spray as described above whereas the data on pod infection were recorded at each picking by examining 500 pods/plot. The yield data on green pods/plot were also recorded and the net profit was worked out.

RESULTS AND DISCUSSION

In both the years, all the spray schedules reduced the per cent disease index, infection rate and RAUDPC in comparison to control. Seven days spray interval gave significantly better control than the spray application at 15 days interval. During 1988-89 spray application of Streptocycline alone or in combination with Blitox 50 WP at 7 days interval converted a commercially useless crop into the one with an acceptable level of disease control (Table 1). Spray application at 15 days interval during 1988-89 could not contain the disease as the spray application at 7 days interval did (Fig 1). Spray application immediately after the disease appearance proved more efficaceous in restricting the disease than the spray application late in the season when the disease had already established. Thus, regular sprayings immediately after the disease appearance are necessary to keep the disease under check. Similar observation was also made by TAYLOR & DUDLEY (1977) in a study on the control of halo blight of beans (*Pseudomonas phaseolicola)* with streptomycin sulphate.

Table 1. Effect of chemical sprays at 2 intervals on bacterial blight severity and pod yield

Treatment	Disease Index* (%)		RAUDPC	Infection rate	Yield (Q/Ha)	Net Profit (Rs./Ha)
	1988	1989	1988	1988	1989	1989
SS-1A	18.71A	0.65A	0. 2559A	0.0105A	203.67A	9652
SS-1B	41.40B	2.35B	0.3930B	0.264B	198.00AB	4633
SS-2A	20.20A	0.62A	0.2609A	0.0105A	204.33A	9156
SS-2B	31.55B	1.24AB	0.3482B	0.0258AB	182.66BC	5583
SBS-A	19.73A	0.77A	0.2464A	0.0127AB	202.00A	8870
SBS-B	36.22B	2.03B	0.3781B	0.0429C	177.66C	4682
Control	85.40C	39.60C	0.7780C	0.0717D	150.00D	

SS-1 and SS-2: Spray of Streptocycline 100 and 200 ug/ml, respectively; SBS: Spray of Streptocycline (100 µg/ml) + Blitox (0.2%); A: 7days interval; B: 15 days interval; *Recorded 15 days after last spray; RAUDPC Relative area under disease progress curve; Mean separation within a column by DMRT at 5% level.

Seed dip in Streptocycline (250 μg/ml) for 2 and 4 h reduced the disease incidence by 95 per cent with out reducing the seed germination (Fig 2) but these treatments failed to protect the crop upto harvesting time. TAYLOR & DYE (1976) also obtained a similar level of control of *P. syringae* pv. *pisi* from pea seeds with streptomycin sulphate (0.25 %).

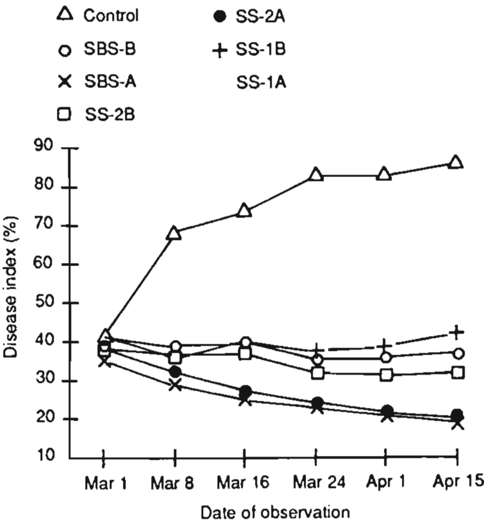

Fig. 1. Line graph showing the effect of chemical sprays on the progress of bacterial blight

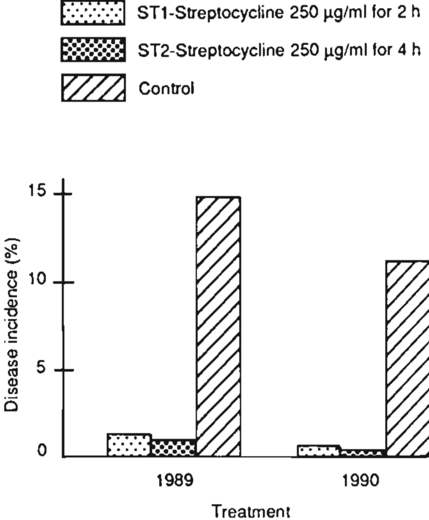

Fig. 2. Effect of different seed treatments for the control of P. *syringae* pv. *syringae* from pea seeds.

Combination of seed treatments with spray application of Streptocyclinewere significantlybetter thanthe seed treatment orspray alone (Table 2). Sprayapplication at7daysintervalgave significantly better control than applications at 15 days interval as observed in the previous experiments. Combination of seed treatment with Streptocycline (250 μg/ml) for 4 h followed by its application @ 100 μg/ml at 7 days interval was most effective and gave 96 per cent reduction in leaf infection and cent per cent control of pod infection. The green pod yield and net profits were also maximum in combinations followed by spray aplication at 7 days interval and seed treatments. The net profit for the combinations, foliar sprays at 2 intervals and seed treatments ranged between Rs.11638 to 16174, 7050 to 13504 and 7104 to 8454 during 1989-90, respectively. The corresponding figures for 1990-91 are Rs.11160 to 18406, 8666 to 13266 and 9000 to 10332, respectively.

Table 2. Effect of Streptocycline seed treatment durations and spray intervals alone and their 4 combinations on the bacterial blight intensity and green pod yield

Treatment	Disease index* (%)		Pod infection (%)		Yield (Q/Ha)		Net Profit (Rs./Ha)	
	1989	1990	1989	1990	1989	1990	1989	1990
ST-2h	37.7E	14.8E	9.3F	7.3D	163.7E	171.6D	7104	9000
ST-4h	31.5E	15.1E	7.6E	6.6CD	170.5DE	178.3CD	8454	10332
SS-A	7.2BC	5.1B	5.9D	1.6B	201.0BC	195.0B	13504	13266
SS-B	15.0D	9.4D	8.3EF	5.0C	166.3E	170.0D	7050	8666
ST-2h + SS-A	2.1A	0.9A	0.0A	0.0A	214.5A	218.7A	16174	18406
ST-2h + SS-B	6.2BC	8.8CD	5.3D	2.1B	190.0BC	182.5C	11754	11166
ST-4h + SS-A	2.9AB	0.8A	2.0B	0.0A	203.6AB	213.3A	14000	17332
ST-4h + SS-B	9.7CD	7.7C	3.6C	1.6B	186.4CD	182.6C	11638	11200
Control	55.7F	38.0F	21.3G	11.1E	128.0F	126.6D		

ST: Seed treatment with Streptocycline (250 ug/ml) for 2 h and 4 h durations;SS: Spray application of Streptocycline (100 ug/ml); A: 7 days interval; B: 15 days interval; * Recorded 15 days after last spray following 0-5 scale; Mean separationwithin a coloumn by DMRT at 5% level

From the present study it is clear that the seed treatment with Streptocycline (250 µg/ml) for 4 h should be followed by its spray application at 7 days interval for an efficient management of bacterial blight of pea.

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Detection of bacterial soft rot pathogens on seed-producing cabbage plants and their control

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ABSTRACT

For some years in North-east Germany great losses in seed-producing cabbage plants by soft rotting have been observed. Out of 342 cabbage samples analyzed from 1987 to 1990, 65% contained soft-rotting bacteria. From the pectolytic bacteria which were found, 53% belonged to *Erwinia carotovora*, 37% have been identified as pseudomonads and 10% as bacilly. The ratio : *Erwinia/Pseudomonas* isolates increased from 10/90 before storing to 70/30 during the seed harvest time in the second cultivation year.

From 65 chemical compounds under test especially $KMnO_4$ and the fungicide copac E considerably checked disease development under practical conditions. Some antagonistic bacteria also reduced the soft rot incidence.

KEYWORDS

Soft rot, cabbage, seed production, *Erwinia carotovora*, pectolytic pseudomonads, pectolytic bacilly, prevention, chemical control, biological control.

INTRODUCTION

For some years great losses at cabbage heads, by soft rotting during the storing period and at seed-producing plants in the field, have been observed in the traditional cabbage cultivation region in N.E. Germany having belonged to the former GDR. High damages at cabbage stocks were also stated by other authors in different countries (KELLER & KNÖSEL, 1980 ; KIKUMOTO & OMATZUZAWA, 1981 ; GIESSMANN & SEIDEL, 1984 ; CEPONIS *et al.*, 1987).

As a pre-requisite of a successful control, the occurrence of soft rot at the heads provided for seed-producing and the dynamics of the soft-rotting bacteria in cabbage plants were studied from 1987 to 1990. Furthermore, different possibilities to reduce soft rot incidence have been tested.

MATERIAL AND METHODS

For monitoring the soft-rotting bacteria samples from diseased head and stalk tissue, taken before, during and after storing, before planting and during the seed-producing year, were transferred on to the surface of potato tuber halves to enrich tissue macerating organisms. Tuber tissue having been soft-rotted was streaked upon selective media for pectolytic erwinias (STEWART agar), pseudomonads (FPA) and bacilly (thioglycollate agar with pectate overlay). A part of the samples was heated (70 to 80°C , 10 min) to select the spore-forming bacteria.

For testing control measures, a simple bio-assay using cabbage stalk slices as substrates for a virulent isolate of *Erwinia carotovora* subsp. *carotovora* (No 2270) was developed. Furthermore, cabbage heads were sprayed by test preparations before storing and after planting. In the same way some antagonistic bacteria isolated from different plants could be tested for their disease reducing effect.

RESULTS

Analysis of cabbage plants on soft rot incidence

The test of 342 cabbage samples from 1987 to 1990 for the occurrence of soft rot brought the following results.

The proportion of cabbage heads with soft rot symptoms raised in the course of the storage period. In contrast, the number of stalks with symptoms did not increase till the planting (Table 1). A high percentage of the cabbage samples induced maceration in the potato slice test.

The pectolytic bacteria occuring in the cabbage tissues showed a remarkable fluctuation. Before storing pectolytic pseudomonads distinctly prevailed. During the storage phase and especially in the following fructification period the frequency of *Erwinia carotovora* considerably increased. This happened in both places (NE and Central Germany) which we have studied and in both test years. However, in 1988-1989 such a dominance of *Erwinia* as in the first investigation period was not reached (Fig. 1). With exception of the seed harvest period the ratio *Erwinia*/*Pseudomonas* was very similar at both test sites.

Occurring for the first time, in the second vegetation year (1989), together with *Erwinia carotovora*, were some pectolytic *Bacillus* strains.

Table 1 : Occurrence of cabbage plants with soft rot symptoms Groß Brütz 1988/1989

Stage/Date	Soft rot incidence* Head	Soft rot incidence* Stalk	Number of samples Tested	Number of samples Containing soft rot bacteria **
Before storing (22-12)	31	0	20	1
During storing (6-2)	45	4	20	17
Before planting (17-4)	94	0	20	20
During seed-producing period (8-8)	-	12	20	17
During seed harvest (3-10)	-	31	35	30

* Percentage for nearly 100 heads tested per date

** Tested at HR Aschersleben

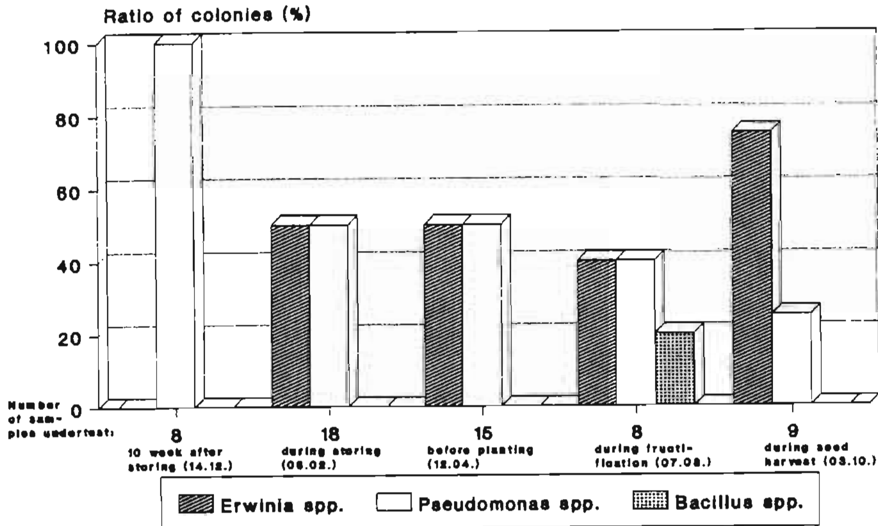

Fig 1 : Occurrence of pectolytic bacteria on cabbage plants with soft rot symptoms during storage and seed-producing period, Groß Brütz 1988/1989

In the autumn of the third year of inquiry (1989) no soft rotted cabbage plants were detected. That may have been a consequence of the dry conditions in this year. Nevertheless, during and after storage many pectolytic strains of *Erwinia* could be isolated from the heads.

Experiments for controlling soft rot in cabbage plants

In the cabbage stalk slice assay (out of 65 preparations under test), especially streptomycin, nourseothricin, kasumin liquid and cupric sulphate (1%), slaked lime, the copper-containing fungicide kocide 101, bronopol and two test preparations remarkably diminished the soft rot by prophylactic application (Table 2).

RAUSCHE *et al.* (1980) and GEESON & BROWNE (1989) also achieved a successful control of cabbage rots by application of fungicides. BORROMEO & ILAG (1984) reported on good results against bacterial soft rot of cabbage by lime treatment.

In contrast, the control effect of some antagonistic bacteria only reached an efficiency between 50 and 60%.

Table 2 : Effect of some chemical preparations on *Erwinia carotovora* subsp. *carotovora* tested by a cabbage stalk slice bio-assay.

preparation	(%)	index of disease **	efficiency***
control (inoculated)		83.3	-
Hg_2Cl_2	0.1	24.7	70.3
$CuSO_4$	1.0	33.3	61.0
$KMnO_4$	0.5	57.1	31.4
bronopol	0.1	23.8	71.4
streptomycin	0.025	20.0	76.0
nourseothricin	0.1	4.4	94.7*
kasumin liquid	0.05	19.1	77.1*
preparation A	0.5	14.6	82.5*
preparation B	1.0	33.3	60.0

* Significantly different from the control variant ($\alpha = 0,05$)
** After TOWNSEND & HEUBERGER (1943)
*** After ABBOTT (1925)

Under practical conditions the application of potassium permanganate and the copper-containing fungicide copac E allowed to obtain most of the plants ripe seeds and symptomless plants. Nourseothricin and kasumin liquid reduced the incidence of soft rot only slightly under practical conditions. Contrary to copac E, potassium permanganate had a good protective effect in all three test years.

Soft rot losses could also be decreased by treatment of the cabbage heads by storing them together with antagonistic bacteria.

DISCUSSION

Soft rot is a frequent disease during cabbage seed production in Germany, especially when susceptible varieties are cultivated. Therefore, a careful treatment during the vegetation period and harvest and dry aerobic storing of the heads are necessary measures to limit the disease.

The dominance of pectolytic pseudomonads after harvest indicates that these organisms are probably natural inhabitants of the plant surface. These observations explain why the now usual cross-cutting of heads for facilitating the sprouting of inflorescences raises the danger of infection by soft-rotting bacteria.

A treatment of the heads before storing with suitable bactericidal compounds can remarkably protect them from bacterial soft rots. Under practical conditions potassium permanganate showed the best protective effect.

Some antagonistic bacteria may be also able to reduce soft rot development effectively.

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Control assays of *Xanthomonas campestris* pv. *pelargonii* by heat treatment and desinfectants

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Current *Pelargonium* screenings by bacterial isolation and ELISA led to a systematic investigation of different control measures.
In micropropagation tests using *Pelargonium x hortorum* "Isabell" the effect of apical explant sizes (0.2-0.5mm ; 3.0-5.0mm) on the transmission of latent infections was studied: up to 28% of symptomless plantlets issued from artificially infected cuttings were *Xanthomonas campestris pv. pelargonii* positive. In the following the suitability of heat treatments was investigated. The viability of bacterial suspensions (10^8 cfu/ml) exposed to temperatures higher than 40°C rapidly diminished. Incubations of in vitro plantlets and potted plants also led to a certain decrease of bacterial infections within this range of temperature. A regeneration of heat-treated plants was observed. Finally seven different desinfectants especially used in greenhouse applications were tested. Taking into account the different doses and incubation times the product M&ENNO ter forte (Menno Chemie, Germany) was found to be the most suitable.

Keywords : *Xanthomonas campestris pv. pelargonii*, *Pelargonium x hortorum* micropropagation, heat treatment, desinfectants.

1.Introduction

Pelargonium is one of the most important ornamental crops in Austria. As in other countries the major disease problems occur by *Xanthomonas campestris pv. pelargonii* (Brown) Dye, the bacterial wilt (DIGAT, 1987; REUTHER, 1988). Voluntary greenhouse screenings at the basis of ELISA

tests revealed infection rates up to 30%. Therefore systematic investigations on control measures at different *Pelargonium* production steps were started. The objectives of the present study were:

a.) to follow the possible transmission of bacterial infections through micropropagation

b.) to evaluate the suitability of heat treatments as control measure

c.) to check the efficiency of commercially available desinfectants for cultural practice.

2. Materials and methods

All assays were performed with *Pelargonium x hortorum* cv. "Isabell" and *Xanthomonas campestris pv. pelargonii* strain 50857 (Deutsche Sammlung von Mikroorganismen).

2.1 Transmission of bacterial infections by apical explants excised from infected cuttings.

Cuttings were artificially infected by dipping in bacterial suspensions (10^4 cfu/ml) for 20 minutes, cultivated in greenhouse for 2 weeks and kept 1 week at 4°C prior to explant excision. Apical explants had two sizes: 0,2-0,5 mm and 3,0-5,0 mm. They were cultivated on a modified Murashige and Skoog medium (MURASHIGE & SKOOG, 1962) at 25°C and 16h light period for 14 weeks.

2.2 Heat treatments of bacterial cultures, artificially infected in vitro plantlets and potted plants

Suspensions of strain 50857 (10^8 cfu/ml) in 0,1 M phosphate buffer overlayed with steril oil were submerged at 30°C, 40°C, 45°C and 50°C in water. Samples were taken in regular intervals and plated on yeast-pepton-glucose-agar (YPGA).

In vitro plantlets were infected in the upper part of the stem by a syringe (suspension of 10^7 cfu/ml) and kept 3 days at 25°C prior to heat treatment. Thermotherapy was performed at 40°C for 40h and 60h. Surviving plantlets were screened for bacterial infections 4 weeks after heat treatment.

Stems of three months old potted plants were inoculated (10^7cfu/ml) and kept 3 days at 25°C prior to heat treatment. Thermotherapy was performed at 45°C and 90% relative humidity for 7h. Plants remained at least for 3 days at 25°C before bacterial screening.

2.3 Diagnosis of *Xanthomonas campestris pv. pelargonii*

Whole in vitro plantlets were homogenized in 0.5ml steril water. From potted plants 1 g of infected stem was dissected in 4.0 ml steril water. The suspensions were both plated directly on YPGA, and enriched in yeast-pepton-glucose (YPG). When atypical colonies occurred, ELISA tests were made additionally (Sanofi).

2.4 Desinfection of scalpels

Scalpels, simulating the use of contaminated knives, were successively immersed in bacterial suspensions (10^7 cfu/ml, 2 minutes), in desinfectant solutions (30", 1.0 min, 5.0 min, 10,0 min) and finally in steril water (20"). The final suspensions were plated on YPGA. Products with the following active agents were tested: Quarternary ammonium compounds: M&ENNO ter forte (Menno Chemie) and DC-Gol (Thermochema), organic acids: Venno Cycla 2 (Menno Chemie), Venno Terra Spray (Menno Chemie), organic acids and alcohols: Menno quick (Menno Chemie), aldehydes and alcohols: Agrodor (Agro Fattinger); Natriumhypochlorite; Ethanol.

3. Results

3.1 Effect of apical explant sizes on the transmission of strain 50857

Approximately all shoot tips (3.0-5.0 mm) excised from infected cuttings died within a culture period of 14 weeks, whereas 27% of the meristems survived. From these remaining explants up to 14% were *Xanthomonas campestris pv. pelargonii* positive (table 1). Even in preliminary investigations the infection rate was 28%.

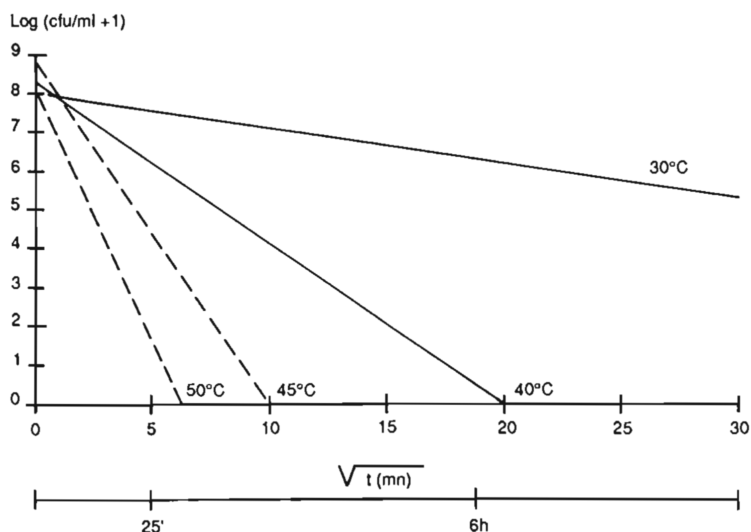
Table 1: Transmission of *Xanthomonas campestris pv. pelargonii* through apical explants

explant size (mm)	Number of explants tested/ excised	total number of infected plants	Number of explants surviving 14 weeks	%	Number of infected surviving explants
3.0-5.0	156/156	147	3	1,9	1
0,2-0,5	112/156	56	42	26.9	6

3.2 Effect of heat treatments

The viability of strain 50857 rapidly diminished when exposed to temperatures higher than 40°C. At 30°C all bacteria were killed within 7 days and at 50°C within 40 minutes (Figure 1).

Figure 1: Thermosensitiviy of *Xanthomonas campestris pv. pelargomii*



A low number of in vitro plantlets survived heat treatments at 40°C (table 2). After an exposure for 60h no bacterial infection was detected in surviving plants. The incubation of potted plants at 45°C led to a partial elimination of bacterial infections. In 41% of stems no bacteria were detected, in 9% the bacterial contaminations were in the order of 10^1 to 10^3 cfu/ml and in 50% of stems the number of bacteria was 10^4-10^7 ćfu/ml. In

infected, not heat treated plants, 91% of stems were contaminated with 10^6-10^7 cfu/ml.

Table 2: Effect of heat treatments on artificially infected in vitro plantlets

temperature °C	time h	initial number of infected plantlets	number of plantlets surviving 4 weeks	%	number of infected surviving plantlets	%
40	40 60	20 23	7 6	35 26	5 0	71 0
25		20	0	0	0	0

3.3 Effect of desinfectants

Different desinfectants, M&Enno ter forte, Venno Cycla 2, Venno terra spray and Menno Quick had a better effect than ethanol (table 3).

Table 3: Desinfection of contaminated scalpels

Desinfectant	conc. (%)	incubation time 30"	1'	5'	10'
M&Enno Ter forte	0,001 0,1 0,25	+++ - -	+++ - -	+++ - -	++ - -
DC-Gol	1,0	+	+	-	-
Menno Quick	100	-	-	-	-
Agrodor	0,5 2,0	+++ +++	+++ +++	++ +/-	+ -
Venno Cycla 2	1,0	-	-	-	-
Venno Terra Spray	100	-	-	-	-
Ethanol	70	+/-	+/-	-	-
Na-hypochlorite	0,5	-	-	-	-
H2O		+++	+++	+++	+++

-..0 cfu/ml, +..<102cfu/ml, ++..102-104cfu/ml, +++..>104cfu/ml

5. Discussion

The present investigations yield the following results:

a) as already mentioned by Reuther (REUTHER, 1988) infections can be transmitted through apical explant culture, and obviously the risk of transferring latent infections increases when small explant sizes (0.2-0.5 mm) are used.

b) like in other bacterial systems heat treatments induced reduction of *Xanthomonas* infections and may be used as control measure (KECK et al., 1990). However due to the plant thermosensitivity the methods should be improved.

c) Commercially available desinfectants recommended against *Xanthomonas campestris pv. pelargonii* differ not only in their active chemical compound but also in their bactericid effect, also shown by Burki (BURKI, 1974). Therefore an official registration of desinfectants used in horticulture was initiated.

Finally we should remark that all three different measures had only a limited impact and cannot replace the improvement of bacterial screenings and resistant cultivars (DUNBAR & STEPHENS, 1989).

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Effect of hot water treatment on *Xylophilus ampelinus* in dormant grape cuttings [1]

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ABSTRACT

The effect of hot water on the survival of *Xylophilus ampelinus* both in different suspending media and in artificially and naturally infected grapevine canes of the cv. Sultana was investigated. The suspending medium played an important role on the survival of the bacterium to the exposure at different temperatures. Suspending media rich in nutrients supported the survival. The bacterium tolerated 90 min exposure at 45°C when Nutrient broth or Yeast Salt media were used while it was killed after 20 min exposure when suspended in half strength Knop's solution. Exposure at 50°C for 5 min killed a bacterial population of 7.5 x 106 cfu/ml irrespectively of the medium used.

Artificially inoculated canes with approximately 3.2 x 10^8 cfu/ml were freed from the bacterium, or its population was reduced to below the level of detection after exposure at 45°C for 20 min. However, it does not guarantee the eradication of the bacterium.

From the results obtained, it could be concluded that the hot water treatments offers a simple, effective, economical and environmentally safe method for eradicating *Xylophinus ampelinus* from dormant grape cuttings.

The method could be recommended to prevent the introduction of the disease, through the importation of propagating material, in countries where this disease is not present.

KEYWORDS

Xylophilus ampelinus, grapevine, hot water treatment, dormant cuttings, eradication.

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INTRODUCTION

Bacterial blight or bacterial necrosis of grapevine caused by *Xylophilus ampelinus* (Panagopoulos) Willems is a serious, chronic vascular disease of grapes. In Greece the disease is endemic in the island of Crete, where it was described by Sarejanni in 1939. Its etiology was clarified by Panagopoulos in 1969. The causal agent, *Xanthomonas ampelina*, was reclassified as *Xylophilus ampelinus* by Willems et al. in 1987. The most characteristic symptom of the disease is the formation of cankers on branches canes, young shoots, and petioles. Diseased plant parts upon longitudinal section show a red-brown discoloration of the xylem tissues. However, the extent of symptom development and the type of symptoms vary and depend on different parameters as the cultivar, the environmental conditions, and the time of infection.

Canes which were infected later in the growing season may not show any visible symptom, or vascular discoloration. These canes which are latently infected are the most significant sources for diseminating the pathogen in long distances. It has been shown (PANAGOPOULOS, 1987) that a proportion of 32-50% of apparently healthy canes collected from an infected vineyard of the cv. Sultana, were infected by the pathogen.

To prevent the spread of the disease in an unaffected region it is very important that all planting and grafting material to be pathogen-free. Since visual examination of the propagating material does not cover the latently infected ones the use of hot water to eradicate the pathogen from artificially and naturally infected dormant canes was investigated.

MATERIALS AND METHODS

Bacterial cultures

Two strains of *X. ampelinus* from our collection were used. The strain BPIC 48 which is the Type strain and the BPIC 620. The bacteria were maintained on NA (Nutrient agar, Difco) slants and for inoculum preparation they were grown on GYCA (Galactose,

Yeast, Carbonate, Agar) at 26°C.

Thermosensitivity of *Xylophilus ampelinus*

The effect of exposure time at 45 and 50°C on the survival of the bacterium was studied with two methods. a) From an initial suspension containing $1.8X10^{3}$ cfu/ml decimal dilutions up to 10^{-}[illegible] were made in test tubes containing NB (Nutrient Broth, Difco) or YS (Yeast Extract, Salts). Six sets of tubes were placed in a water bath at 45°C and 50°C. One set of tubes was removed after exposure for 10, 20, 25, 30, 60 and 90 min, at 45°C and 5, 10, 15, 20 and 30 min at 50°C. One set of tubes was used as control (no heat treatment). The tubes, after the exposure, were incubated at 26°C for 10 days and checked for bacterial growth (turbidity). From the tubes showing growth a loopful of suspension was streaked onto NA to check possible contamination. b) Decimal dilutions up to 10^{-7} were made in half strength Knop's solution. From each dilution 1 ml was placed into lyophilization tubes and six sets of the tubes were placed each time into a water bath at 45° and 50°C. One set of tubes was removed after exposure of the time scheduled. 50 μl of suspension was removed from each tube, streaked on NA containing petri dishes and incubated at 26°C for 10 days. The dishes were checked for colonies of *Xylophilus ampelinus*.

Artificial inoculation of plant material

One year old canes of the cv. Sultana were collected from different locations at different times. The canes were cut in 40 cm long pieces and kept at 4 to 6°C in a refrigerator into polyethylene bags until use. The artificial inoculation of the canes was performed by infiltration with a bacterial suspension in half-strength Knop's solution containing $5X10^{6}$ cfu/ml. The infiltration was achieved either by sucking or by forcing the bacterial suspension to pass through the cane. In the first case the uper end of the cane was connected to the inlet tube of a vacuum pump while the base of the cane was immersed into the bacterial suspension. One ml of suspension was sucked. In the second case the base of the cane was connected with a syringae through a thick-walled elastic tube and the bacterial suspension was forced to pass through the cane, until a drop was formed at the end of the cane. This method was developed by Dr A.S.

Table 1. Lethal effect of hot water treatment at 45°C and 50° on dilutions of *Xylophilus ampelinus*[1] in different suspending media.

Medium	Highest population of bacteria killed at			
	45°C		50°C	
	Cfu/ml	Treatment time[2]	Cfu/ml	Treatment time[2]
NB (Difco)	$6,0x10^{6}$	60 min	$7,5x10^{6}$	5 min
YS	$6,0x10^{6}$	90 min	$6,0x10^{7}$	5 min
(1/2) Knop's solution	$1,7x10^{6}$	20 min	$1,7x10^{7}$	5 min

1. Five day old growth on GYCA (Galactose, Yeast, Carbonate, Agar) slants of the strains BPIC 48 and 620 were each suspended in 5ml of water, and combined (initial population $6x10^{8}$ cfu/ml).

2. The time required for the suspending medium to reach the test temperature was checked by a thermometer placed in a test tube containing the suspending medium.

Table 2. Effect of heat treatment at 45 and 50°C for 20 and 30 min on isolation of *X. amp.* from treated canes.

Treatment	45°C			50°C			Control
	No of cuttings tested	No of cuttings with *X.a*/ total		No of cuttings tested	No of cuttings with *X.a*/ total		No of cuttings with *X.a*/ total
		20	30		20	30	
Artificial inoculated	95	0/39	0/56	132	0/87	0/45	21/42
From diseased vineyards	18	0/18	–	69	0/46	0/23	7/51
Infiltrated with Knop's solution	–	–	–	21	0/6	0/15	0/51

Table 3. Effect of heat treatment at 45 and 50°C for 20 and 30 min on infection and rooting of cuttings.

Treatment	45°C				50°C				Control	
	No of rooted per total		No of healthy cuttings per total		No of rooted per total		No of healthy cuttings per total		No of rooted per total	No of healthy cuttings per total
	20min	30min	20min	30min	20min	30min	20min	30min		
Artificial inoculated	13/15	21/29	11/13	21/21	34/45	17/31	34/34	17/17	13/30	2/13
From diseased vineyards	-	-	-	-	3/27	12/23	9/9	12/12	3/5	3/3
Infiltrated with Knop's solution	-	-	-	-	-	9/10	-	9/9	19/20	19/19

Alivizatos in our laboratory.

Hot water treatments

The cuttings were grouped according to the treatment they had received and submersed in the water bath in groups of 50. No change in the temperature was observed when the cuttings were submersed. A time interval of 5 min was left for the temperature into the tissues to reach that of the water bath. The cuttings were treated at 45°C and 50°C for 20 and 30 min. After treatment the cuttings were stratified in sand and subsequently part of them were potted for rooting and the remaining were used to make isolations in order to check the presence of *X. ampelinus*. The isolations were made from the middle internode of each cutting.

RESULTS AND CONCLUSIONS

The results are summarized in tables 1,2 and 3. From the results obtained it could be concluded that:
1) *Xylophilus ampelinus*, in suspension, tolerated 45°C at time more than 1 1/2 hours in YS medium. There is an evidence that the suspending medium has some effect on the heat tolerance. 2) The infiltration methods used for artificial inoculation of dormant grape canes proved efficient. Most of the inoculated cuttings did not show any visible symptom on the first year after planting. Typical symptoms of the disease are obvious the second year but not in 100% of the inoculated canes. 3) Hot water treatment at 45°C for 20 min does not guarantee eradication of *Xylophilus ampelinus* from dormant grape canes. 4) Hot water treatment at 50°C for 20 min seems to be effective in eradicating the pathogen from infected dormant canes. Treatment at 50°C for 30 min did not have any lethal effect on the dormant grapevine tissues.

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Green-crop-harvesting and infestation of seed potato tubers with *Erwinia* spp. and perspectives for integrated control

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ABSTRACT

Recently, green-crop-harvesting (GCH) of seed potatoes was developed as an alternative for both chemical haulm destruction (CHD) and haulm pulling (HP). With GCH aerial parts are removed, after which tubers are lifted, replaced on a bed and covered by soil. The availability of the tubers on the lifter offers an opportunity to provide them with biocontrol agents before covering up.

First results showed that numbers of viable *Erwinia chrysanthemi* (Ech) on seed potatoes did not differ significantly between CHD, HP and GCH. In an exploratory field trial on the biocontrol of Ech, promising results were obtained with the application of fluorescent *Pseudomonas* spp., *Gliocladium* spp. and *Trichoderma* spp. at the first lifting with GCH.

INTRODUCTION

To prevent virus infection, Dutch growers of seed potato crops regulatorily have to kill the haulms shortly after mass flights of virus-transmitting aphids begin. The common methods to kill green potato haulms is to pulverize the foliage and then either to pull the haulms or to spray with a herbicide. Black scurf caused by *Rhizoctonia solani* is an important problem of seed potato production. This disorder is strongly promoted by chemical haulm destruction and to a lesser extend by haulm pulling.

Research on harvest methods and prevention of black scurf has led to a green-crop-harvesting (GCH) method (BOUMAN *et al.*, 1990). With GCH aerial plant parts are removed, tubers are lifted, replaced on a bed and covered by soil. As tubers are shortly above ground, an opportunity is offered to treat them with antagonistic organisms and/or chemicals.

GCH implies the lifting of immature tubers, which are prone to damage. Consequently there was a concern for a possible risk of increased problems with

blackleg.

This paper describes the effects of harvesting methods and the application of selected biocontrol agents with GCH on the infestation of tubers with *E. chrysanthemi* and other soft rot bacteria.

MATERIALS AND METHODS

During the 1991 growing season, field experiments on the infestation of offspring tubers with *E. chrysanthemi* (Ech) and other soft rot bacteria were performed in seed potato crops on a light clay soil of "De Oostwaardhoeve" at Slootdorp.

Experiment 1: In a three-factor experiment with cv Mondial the effect of chemical haulm destruction (CHD), haulm pulling (HP) and green-crop-harvesting (GCH), and hardening period (9 and 44 days) on infestation was assessed in plots with and without enhanced contamination with Ech.

Experiment 2: In an exploratory trial (check-plot design; cv Agria), the effect on infestation of the following procedures was assessed: GCH, enhanced contamination with Ech and application of biocontrol agents at the first lifting. Tubers were harvested 14 days after haulm destruction. Biocontrol agents applied were:

- fluorescent *Pseudomonas* spp. (120 ml/m with 6.0×10^{11} colony forming units (cfu's) of a 1:1:1 mixture prepared with 4-days-old trypticase soya agar (BBL) cultures of *P. putida* strain WCS 358 and two isolates of *P. fluorescens* obtained from potatoes grown at "De Oostwaardhoeve");
- *Gliocladium* spp. (120 ml/m with 7.2×10^{10} spores of a 1:1 mixture prepared with 14-days-old cultures of *G. roseum* isolate 1813 and *G.* cf. *nigrovirens* isolate 1815 grown on wheat grains);
- *Trichoderma* spp. (120 ml/m with 2.4×10^{11} spores of a 1:1:1 mixture prepared with 14-days-old cultures of *T. harzianum* isolate 1812, *T. viride* isolate 1811 and *Trichoderma* sp. isolate IVT 10 grown on wheat grains).

As a source for enhanced contamination, tubers induced to rot by Ech (IPO strain 502), were placed (one tuber per two meter ridge) on the front side of the lifter at the first lifting with GCH. For plots without enhanced contamination, non-inoculated tubers were used instead. With CHD and HP, inoculated and "control" tubers were burried in the ridges after pulverization of the foliage.

Infestation of tubers with Ech and other soft rot bacteria was assessed after winter storage. For each experimental entity, each of three 50-tuber-samples were peeled for five minutes by dry abrasion in a potato peeler (model M5; Imperial Machine Company (Peelers) Ltd.). Serial dilutions of sap obtained from peel pulp by vacuum extraction were mixed with PT agar medium (BURR & SCHROTH, 1977) at 46°C in

24-well tissue-culture plates to perform immunofluorescence colony-staining (IFC; VAN VUURDE & ROOZEN, 1990) for Ech, and also plated on a double layer crystal violet pectate medium (DLCVP; PEROMBELON & BURNETT, 1991) to count pectolytic bacteria. With IFC, bacterial colonies were stained with FITC-conjugated antibodies against IPO strain 502 of Ech. The colonies stained with the anti-Ech serum in PT (IFC-positive) and those forming deep cavities on DLCVP were counted.

Statistical analysis was carried out after logarithmic transformation of the numbers of IFC-positive colonies and pectolytic colonies on DLCVP.

RESULTS

Results of experiment 1 are presented in figure 1. Irrespective of the harvesting method and hardening period, numbers of IFC-positive cfu's were higher when harvested after enhanced contamination. Compared to CHD and HP, with GCH numbers of IFC-positive cfu's tended to be slightly lower. Differences for the overall effect of the enhanced contamination on the level of tuber infestation with Ech were significant ($P<0.05$), whereas differences due to the harvest method and hardening period were not significant. Differences of numbers of pectolytic cfu's due to the harvest method, hardening period and enhanced contamination were not significant.

Following the application of the biocontrol agents (experiment 2) reductions in numbers of IFC-positive cfu's (4-10 times) as well as in numbers of pectolytic cfu's (30-700 times) were significant ($P<0.05$) as compared to the non-treated, artificially inoculated and naturally infestated tubers (Fig. 2).

DISCUSSION

Immature tubers are easily skinned or wounded. Despite considerable adaptation of diggers used for first lifting with GCH, some damage of tubers is unavoidable. As wounds are avenues of entry for soft rot Erwinias, it was supposed that GCH would lead to a considerable increase of infestation with these bacteria. However, there is practical and experimental evidence that GCH did not lead to marked increases in infestation with *Erwinia chrysanthemi* (present study), or with *E. carotovora* subsp. *atroseptica* and *E. carotovora* subsp. *carotovora* (unpublished results). Under certain conditions after CHD, the decaying foliage on the ridges was found to be a source of contamination for daughter tubers (ROOZEN, 1991). As with GCH this source of contamination is being removed for the greater part, low infestation may be an attribute of the GCH method.

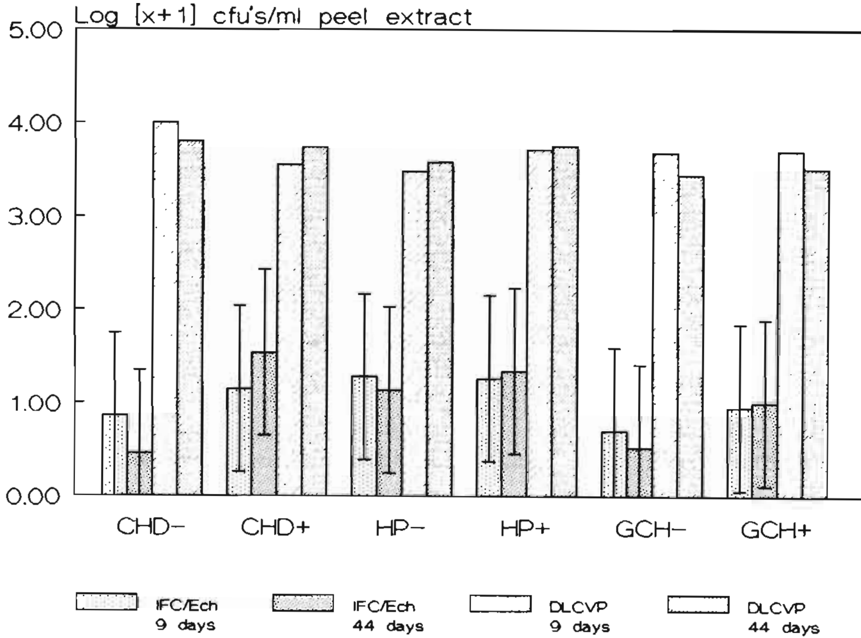

Fig.1. The effect of harvesting method, with chemical haulm destruction (CHD), haulm pulling (HP) and green-crop-harvesting (GCH), and hardening periodes of 9 and 44 days respectively, on numbers of IFC-positive cfu's (IFC/Ech) and other pectolytic bacteria (DLCVP) on seed potatoes from plots with natural infestation (-) and artificial contamination with soft rot diseased tubers. The vertical lines represent the Least Significant Difference for numbers of Ech-positive cfu's.

The application of antagonistic organisms or chemicals at the first lifting with GCH offers good perspectives for integrated control of black scurf (MULDER *et al.*, 1990), gangrene (*Phoma exigua* var. *foveata*) and tuber infection by *Phytophthora infestans* (TURKENSTEEN *et al.*, 1990). As there is an urgent need to control the blackleg syndrome, the effect of application of selected biocontrol agents with GCH on infestation of tubers with Ech was studied. The fluorescent *Pseudomonas* spp. were selected because field experiments demonstrated that several isolates of these plant growth-promoting rhizobacteria show an antagonistic effect on *E. carotovora* (KLOEPPER, 1983; COLYER & MOUNT, 1984; RHODES & LOGAN, 1986; XU & GROSS, 1986). *Gliocladium* spp. and *Trichoderma* spp., antagonists against *P. exigua* var. *foveata* (TURKENSTEEN *et al.*, 1990), were selected because a considerable reduction of bacterial soft rot of tubers was experienced

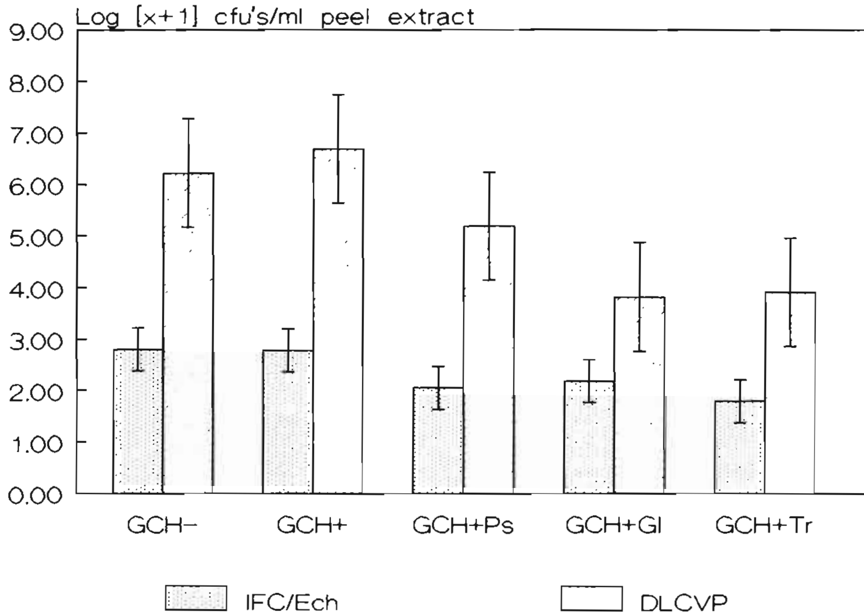

Fig.2. The effect of application of fluorescent Pseudomonas spp. (Ps), Gliocladium spp. (Gl) and Trichoderma spp. (Tr) at first lifting with green-crop-harvesting (GCH) on numbers of IFC-positive cfu's (IFC/Ech) and other pectolytic bacteria (DLCVP) on seed potatoes from artificially contaminated (+) plots. The vertical lines represent Least Significant Differences for numbers of Ech-positive cfu's and other pectolytic bacteria.

after the application of *G. roseum* and *T. harzianum* at the first lifting with GCH in a crop severely affected by Ech (TURKENSTEEN, unpublished results). Moreover, LUGAUSKAS (1961) reported an antagonistic effect of *T. harzianum* on *E. carotovora*. All three groups of biocontrol agents reduced the level of tuber infestation with Ech-positive cfu's and other pectolytic cfu's. Pseudomonads were less effective than *Gliocladium* spp. and *Trichoderma* spp. Therefore, also with regard to blackleg, which disease is shown difficult to manage up till now in seed potato production, GCH offers promising perspectives to deploy integrated control methods.

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Elimination of plant pathogenic bacteria by plant tissue culture; the problem of latent persistence *in vitro*

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Abstract

Although micropropagation has often been thought to automatically eliminate plant pathogenic bacteria from plants, it has recently been implicated in the spread of plant pathogenic bacteria due to latent persistence of the pathogens *in vitro*. To test this hypothesis *Delphinium*, *Aster*, *Iris* and *Rosa* shoot tissue cultures were inoculated *in vitro* with *Agrobacterium tumefaciens*, *Pseudomonas syringae*, *Xanthomonas campestris*, or *Erwinia carotovora*.

Delphinium tissue cultures were killed *in vitro* by all bacterial strains inoculated. However, when *Aster*, *Iris* and *Rosa* tissue cultures were infected, the plant pathogenic bacteria stayed 'latent' (not producing visible growth on the medium or symptoms on the plant) *in vitro*, apart from *Aster* which was killed when infected with *P. syringae* and *Iris* and *Rosa* which were killed by *Erw. carotovora*. Latent infection of *Aster* with *A. tumefaciens* and of *Iris* with *X. campestris* during *in vitro* culture resulted in plants showing the typical disease symptoms (crown galls; leaf spots) *in vivo* after they were weaned into soil.

Key words: micropropagation, *Agrobacterium*, *Erwinia*, *Pseudomonas*, *Xanthomonas*

Introduction

Plant tissue culture methods allow very rapid propagation of plants in limited space in the laboratory and the number of micropropagated plants produced by commercial propagators and plant breeders has increased rapidly over the last thirty years. One of the advantages of micropropagation is the possibility of eliminating diseases caused by fungi, bacteria and viruses from plants and micropropagation is usually defined as the multiplication of plant material under aseptic conditions on artificial media *in vitro*.

Although aseptic conditions are usually implied, plant tissue cultures are often not (or do not stay) aseptic *in vitro*. Furthermore, many bacterial contaminants were shown to stay 'latent' (not producing visible growth in the medium or symptoms on the plant) *in vitro* and can not be detected by visible assessment of plant tissue cultures. Some bacteria which persisted as 'latent' contaminants *in vitro* (eg. *Xanthomonas pelargonii* and *Corynebacterium sepedonicum*, see LEIFERT *et al.* 1991 for a recent review) are known to be plant pathogens *in vivo* (LELLIOTT & STEAD 1987). This and more recent reports from Holland (G. Bartels, personal communication), where imported micropropagated *Aster* uniformly developed crown gall symptoms after weaning on a commercial nursery (which did not have a history of *A. tumefaciens* infestation) persuaded us to examine the effect of plant pathogenic bacteria on a number of different *in vitro* cultured plant species.

Materials and Methods

Aster hybrida 'Pearl Star', *Delphinium* '3072-1', *Iris germanica* 'Amsterdam' and *Rosa* 'Velvet Rosamini' shoot cultures were inoculated with bacterial strains from the National Collection of Plant Pathogenic Bacteria (NCPPB; Harpenden, Herts AL5 2BD, UK; see Table 1 for the NCPPB codes) or *A. tumefaciens* strains Aster 1 and *E. carotovora* strain Iris 1 isolated from contaminated plant tissue cultures at Neo Plants Ltd. Shoots were dipped into a bacterial suspension containing $5x10^5$ cfu/ml before subculture onto new media. After inoculation with pathogenic bacteria shoots were multiplied for 4 month (subcultured onto fresh media every 4 weeks) in irradiated plastic containers (6.5 cm high, 8 cm diameter) containing 50 ml of full strength MURASHIGE and SKOOG's (1962) plant tissue culture medium (MS) and the growth regulators appropriate for the plant species (COOKE *et al.* 1992). Shoots were then rooted *in vitro* by subculture onto rooting media (half strength MS without growth regulators). During *in vitro* multiplication and rooting 10 shoots were planted

per tub and incubated in a growth room at 24°C in the light (15.5 h; 6O $\mu\varepsilon$ of cool white fluorescent light) and 21°C in the dark for 4 weeks before subculture onto fresh media. To confirm the presence of pathogenic bacteria shoot, callus and agar pieces from each tub were sterility tested at every subculture as described in Cooke *et al.* (1992). Bacteria were isolated from *in vivo* plants, which developed symptoms, by the methods described in SCHAAD (1980). Bacteria which were re-isolated from inoculated plant cultures *in vivo* and *in vitro* were identified by API 20E and 20NE test strips.

All rooted plants were weaned individually into 4 x 4 cm compost plugs in polystyrene trays containing 25 plugs. To avoid cross-contamination between plants infected with different pathogens miniature green-houses were created by attaching metal frames to the trays and sealing trays individually in polythene bags. The humidity within the bags was kept at about 95-97 % by spraying plants every 2 days with sterile distilled water. Trays were incubated for 2 months in a growthroom with a constant temperature of 20°C and 16 h light (220 $\mu\varepsilon$ of cool white fluorescent light) per day. Forty plants were then transferred individually into 10 cm diameter pots containing compost and assessed for the development of symptoms for up to 18 month. For *in vivo* pathogenicity testing pot grown plants of the same variety were inoculated with the pathogens and assessed for symptoms using the methods described in LELLIOTT & STEAD 1987).

Results and Discussion

Inoculation of *Delphinium* tissue cultures with each of four species of Gram-negative bacteria (*Agrobacterium tumefaciens*, *Erwinia carotovora*, *Pseudomonas syringae* and *Xanthomonas campestris*) resulted in plant death during growth on multiplication or rooting media *in vitro* (Table 1). *Aster* inoculated with *Ps. syringae* and *Iris* and *Rosa* infected with *Erw. carotovora* also died *in vitro*. All plants which were killed during growth on multiplication or rooting media showed visible bacterial growth in the plant medium. Death of plant tissue cultures after inoculation with *Ps. syringae* and *X. campestris* was associated with unspecific stress symptoms such as chlorosis and necrosis of leaves, while inoculation of *Erw. carotovora* resulted in soft rot symptoms and maceration of the tissue of *Cheiranthus*, *Delphinium* and *Iris*.

All other bacteria inoculated into *Aster*, *Iris* and *Rosa* cultures did not cause symptoms on plants, bacterial growth on the medium or reduction in multiplication

rates or fresh weight gain (except for *Aster* inoculated with *X. campestris* or *Erw. carotovora*; Table 1). However, *Aster* inoculated with *A. tumefaciens*, *X. campestris* or *Erw. carotovora*, *Iris* inoculated with *X. campestris* and *Rosa* inoculated with *P. syringae* and *X. campestris* stayed infected *in vitro* (the bacteria inoculated could be detected on plants throughout the experiment by sterility testing and/or selective media). *Aster* inoculated with *A. tumefaciens* showed an increased fresh weight and had slightly larger calli, but similar multiplication rates. No bacteria could be detected after inoculation of *Iris* with *A. tumefaciens* or *Ps. syringae* and *Rosa* with *A. tumefaciens* and multiplication rates and fresh weight gains remained similar to control plants (Tables 1).

All 'latent' infected plants were weaned successfully, but 6 months after transfer to compost (a time when many micropropagated plants would already have been dispatched by the laboratory and planted in the field or in a customer nurseries) *Aster* inoculated with *A. tumefaciens* had developed crown galls and *Iris* inoculated with *X. campestris* developed leaf spots and necrotic leaves. These symptoms were similar to those observed during pathogenicity testing and *A. tumefaciens* and *X. campestris* strains with the same API 20NE profiles as those originally inoculated could be isolated from the crown galls and leaf spots. These findings confirm earlier results which showed that *Corynebacterium sepedonicum* and *Xanthomonas pelargonii* can stay latent *in vitro* (see LEIFERT *et al.* 1991 for a review) and indicates an important hazard of micropropagation. If plants are not tested for the presence of plant pathogenic bacteria, diseased plants can be propagated and distributed and the inter-continental transport of plants as plant tissue cultures could result in pathogens or new strains of pathogens being introduced into regions or countries where they were not endemic previously.

The finding that *A. tumefaciens* and *Ps. syringae* pv. *delphinii* could not persist *in vitro* after inoculation into *Iris* might be expected, since both bacteria are known not to be pathogenic to this species (LELLIOTT & STEAD 1987). However, the failure

Legend for Table 1

[a] = pH was measured at subculture after 28 days of plant growth; [b] = mean is of 50 plants and 4 subsequent subcultures; [c] = assessed during 4 subsequent subcultures; d = all plants dead; [h] = plant species the bacteria were shown to be pathogenic during *in vivo* pathogenicity testing; [s], plants developed disease symptoms 6 month after weaning; n = no significant difference to uninfected control plants; * = significantly different to control (p=0.05); ** = significantly different to control (p=0.001).

Table 1. Medium pH, multiplication rate and fresh weight gain of plant cultures infected with plant pathogenic bacteria and percentage of shoots visibly infected and positive in infection tests

Bacterial species	Strain code	Plant genus	Medium pH uninfected control plants[a,b]	Multiplication rate *in vitro* (% of control plants)[b]	Fresh weight gain *in vitro* (% of control plants)[b]	Shoots visibly infected during 4 month of multi-plication and subsequent rooting *in vitro* (%)		Shoots giving positive infection test results during multiplication and rooting *in vitro* (%)	
						multiplication[c]	rooting	multiplication[c]	rooting
A. tumefaciens	1650	*Delphinium*[h]	5.9	77**	66**	2-80	100d	20-100	100
	Aster1	*Aster*[h,s]	4.3	96n	124**	0	0	100	100
	1650	*Iris*	4.1	95n	101n	0	0	0	0
	1650	*Rosa*[h]	3.9	97n	92*	0	0	0	0
Ps. syringae	1879	*Delphinium*[h]	5.9	d	d	d	d	100	d
	1879	*Aster*	4.3	91*	106n	0	100d	80-100	d
	1879	*Iris*	4.0	100n	103n	0	0	0- 38	0
	2732	*Rosa*[h]	3.9	94n	92*	0-39	0	100	100
X. campestris	2517	*Delphinium*	5.9	d	d	d	d	100	d
	2517	*Aster*[h]	4.5	73*	65*	0-30	0	100	100
	2517	*Iris*[h,s]	4.0	97n	104n	1-20	0	100	100
	2517	*Rosa*	3.9	99n	100n	0	0	80-100	100
E. carotovora	1860	*Delphinium*[h]	5.8	d	d	d	d	100	d
	1860	*Aster*	4.3	80**	83**	0- 8	45	100	100
	Iris1	*Iris*[h]	3.9	d	d	d	d	100	d
	1860	*Rosa*	3.9	72**	65**	0-46	d	100	d

of *A. tumefaciens* isolate 1650 to persist in *Rosa* is somewhat surprising since the bacterium was shown to be pathogenic *in vivo*. Failure of the sterility test methods used to detect bacteria could be one reason for their apparent non-persistence, but they could also have been eliminated by plant resistance mechanisms which have been shown to prevent growth and persistence of bacterial contaminants such as *A. tumefaciens*, *Bacillus subtilis*, *Ps. syringae*, *Pseudomonas maltophilia*, *Staphylococcus saprophyticus* and *X. campestris* in *in vitro* cultures (LEIFERT *et al.* 1991; COOKE *et al.* 1992).

Further research should therefore investigate the mechanisms which suppress plant pathogenic bacteria *in vitro* and investigate more sensitive serological methods for the detection of plant pathogens in plant tissue cultures to avoid propagation and distribution of infected plant material.

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Sources of resistance to *Clavibacter michiganensis* subsp. *michiganensis, Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato* in the *Lycopersicon* species

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INTRODUCTION

Bacterial canker (*Clavibacter michiganensis* subsp. *michiganensis* - *Cmm*), bacterial spot (*Xanthomonas campestris* pv. *vesicatoria* - *Xcv*) and bacterial speck (*Pseudomonas syringae* pv. *tomato* - *Pst*) are the most widespread and destructive diseases of tomato in Bulgaria. Breeding resistant tomato lines and cultivars is one of the most efficient ways of control. Investigations connected with the search of resistance to bacterial agents in wild species in the genus *Lycopersicon* were conducted. Resistance was discovered in *L. pimpinellifolium* (THYR, 1968 ; PILOWSKY and ZUTRA, 1982 , LAWSON and SUMMERS, 1984), *L. hirsutum* (HASSAN *et al.*, 1968 ; SOTIROVA and BELEVA, 1975 ; KURIYAMA *et al.*, 1974), *L. chilense* (YORDANOV and STAMOVA, 1978 ; SOTIROVA and BOGATZEVSKA, 1988-1990), *L. peruvianum* (LINDHOUT and PURIMAHUA, 1987) to individual agents of bacterial diseases. The studies carried out by BOGATZEVSKA *et al.* (1989-1992) showed the presence of race O *Pst* and tomato and pepper-tomato pathotypes in Bulgaria.

The aim of the present investigation was to look for sources of resistance in various accessions of the sub-genus *Eulycopersicon* and sub-genus *Eriopersicon* of the genus *Lycopersicon*.

MATERIAL AND METHODS

Various accessions from wild species of the genus *Lycopersicon* were tested for resistance to *Cmm*, *Xcv* (tomato pathotype *XcvT*, pepper-tomato pathotypes *XcvPT*), *Pst* race 0 and race 1. The plants were inoculated by the vacuum infiltration method (BOGATZEVSKA, 1988) in the phase 4-5 true leaves for *Xcv* (10^8 cfu/ml) and *Pst* (10^4 cfu/ml). The evaluation of the disease severity was made on the bases of SOTIROVA and BELEVA's scale (1975) for *Xcv* and by CHAMBERE and MERRIMAH's scale (1975) for *Pst*. The inoculation with *Cmm* (10^8 cfu/ml) was made in phase 3-4 true leaves by injection after the method of DE

Table. 1

Resistance of wild tomato species of genus Lycopersicon to some bacterial pathogens

Wild species and varieties	Cmm	Xcv		Pst	
		T	PT	R_0	R_1
SUB-GENUS EULYCOPERSICON					
Lycopersicon esculentum	S	S	S	S	S
L. esculentum ssp. galenii	S	R	S	R	R
L. esculentum var. pyriforme	S	S	S	S	S
L. esculentum var. cerasiforme LA 1205	R	-	-	S	S
L. esculentum var. cerasiforme LA 1207	R	-	-	S	S
L. esculentum var. succentariatum	S	S	-	S	S
L. pimpinellifolium PI 126925	R	R	S	R	S
PI 126444	R	R	R	R	R
PI 306216	R	R	-	-	-
PI 300093	S	R	S	-	-
LA 1594	S	S	S	-	-
LA 1631	R	S	S	-	-
LA1633	R	R	-	-	-
LA 1651	R	-	-	-	-
L. racemigerum	R	R	S	-	-
L. cheesmanii typicum LA 247	S	S	S	S	S
SUB-GENUS ERIOPERSICON					
L. peruvianum PI 128654	S	-	-	S	S
PI 128659	S	-	-	S	S
L. peruvianum GI 1567	-	R	S	R	S
L. peruvianum var. dentatum LA 1566	R	-	-	-	-
L. peruvianum var. gjandulosum LA 1708	R	-	-	R	S
L. peruvianum var. humifusum PI 127828	R	R	S	R	S
PI 127829	R	R	R	R	R
LA 2151	R	R	R	R	R
LA 2334	R	R	R	R	R
LA 2338	R	R	R	R	S
L. chilense LA 456	R	R	S	R	
LA 1164	R	R	S	R	S
L. hirsutum PI 126445	R	R	S	R	S
PI 126927	R	-	-	R	S
L. hirsutum f. glabratum PI 134418	R	R	R	R	S
PI 251305	R	-	-	R	S
LA 407	R	R	R	R	R
L. parviflorum LA 1318	S	-	-	S	S
LA 1326	S	-	-	S	S

JONG and HONMA (1974). The reaction of plants was recorded by LATERROT's scale (1974).

RESULTS AND DISCUSSION

Data of resistance of different accessions sub-genus *Eulycopersicon* and sub-genus *Eriopersicon* to agents of bacterial diseases are represented in table 1. In sub-genus *Eulycopersicon* *L. esculentum* var. *cerasiforme* LA 1205 and *L. pimpinellifolium* PI 126925, PI 126444, LA 1631, LA 1633, LA 1651 showed resistance to *Cmm* and *L. pimpinellifolium* PI 126925, PI 126444 showed resistance to *XcvT*. All except *L. pimpinellifolium* PI 126444 are susceptible to XcvPT. Resistance to race 0 and race 1 of *Pst* is present in *L. esculentum* ssp. *galenii* and *L. pimpinellifolium* PI 126444. In sub-genus *Eriopersicon* accessions from *L. peruvianum* and *L. hirsutum* showed resistance to *Cmm*, *L. peruvianum* var. *humifusum* LA 2151, LA 2334, *L. hirsutum* f. *glabratum* PI 134418 showed resistance to *XcvT* and *XcvPT*, and *L. peruvianum* PI 127829, LA 2151, LA 2334, *L. chilense* LA 456, *L. hirsutum* f. *glabratum* LA 407 showed resistance to *Pst*0 and *Pst*1.

Wild species from sub-genus *Eulycopersicon* were similar in habit to the cultivated tomato and could be easily crossed with it. Although accessions from sub-genus *Eriopersicon* represented valuable sources of resistance to agents of bacterial diseases, their use is limited still, as most of them could not cross or crossed very difficulty with cultivated tomato. The development and application of *in vitro* method in this case could enable to include genes of resistance from wild species of sub-genus *Eulycopersicon* and sub-genus *Eriopersicon* in the genome of cultivated tomatoes.

Our investigation showed that most of the studied accessions are resistant to individual agents of bacterial diseases and represent sources of resistance. Of particular interest are *L. pimpinellifolium* PI 126444, *L. peruvianum* var. *humifusum* PI 127829, LA 2151, LA 2334, *L. chilense* LA 426 and *L. hirsutum* f. *glabratum* LA 407 processing resistance to all three agents of bacterial disease. They could be used as sources of complex resistance to *Cmm, Xcv* and *Pst*.

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Resistance to *Pseudomonas syringae* pv. tomato and *Xanthomonas campestris* pv. *vesicatoria*

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INTRODUCTION

Bacterial speck (*Pseudomonas syringae* pv. *tomato* - *Pst*) and bacterial spot (*Xanthomonas campestris* pv. *vesicatoria* - *Xcv*) of tomato are of economic importance to Bulgaria. *Pst* and *Xcv* are transmitted and survive in tomato seeds (CHAMBERS and MERRIMAN, 1975 ; GOODE and SASSER, 1980) and weeds (VITANOV and BOGATZEVSKA, 1988). It develops as an epiphyte on symptomless tomato plants (BONN *et al.*, 1985), weeds (JONES *et al.*, 1986 ; MC CARTER *et al.*, 1983) and other non-host plants (GOODE and SASSER, 1980) and are preserved in plant debris (CHAMBERS and MERRIMAN, 1975 , GOODE and SASSER, 1980 , JONES *et al.*, 1986). All these features in the biology of the pathogens render control difficult. Breeding resistant tomato lines and cultivars is one of the most efficient way of control (LAWTON and SUMMERS, 1984 ; PITBLADO and KEER, 1980 ; SOTIROVA and BELEVA, 1975 , VITANOV and BOGATSEVSKA, 1988). Race 0 of *Pst* and tomato and pepper-tomato pathotypes of *Xcv* (BOTAZEVSKA and SOTIROVA, 1992 ; BOGATZEVSKA *et al.*, 1989) are widely spread in Bulgaria.

In the present paper the results on resistance of tomato lines to race 0 of *Pst* and tomato and pepper-tomato pathotypes of *Xcv* are presented.

MATERIAL AND METHODS

A collection of 68 tomato lines was tested for susceptibility to race 0 of *Pst* and to tomato *XcVT* and pepper-tomato (*XcvPT)* pathotypes of *Xcv*. Tomato lines consisted of F4 from crossings with participation of wild species *L. minutum* (87610006)*, *L. humboldttii* (87610003), some primitive, small-fruited representatives of *L. esculentum* (83602029, 83602030, 83602027, 83602043), local samples of tomatoes (86E2208) as well as some cultivated varieties (Spectar and Mercury).

* Number in the National Genebank IIPGR, Sadovo.

The plants (50 per line) were inoculated by the vacuum-infiltration method (BOGATZEVSKA *et al.*, 1989) at the stage 2-3 leaves for *Pst0* and 5-7 leaves for *Xcv* (*T* and *PT*). The infiltration was done with suspensions of 36 hour old cultures of *Pst* (10^4 cfu/ml) and *Xcv* (10^8 cfu/ml). The plants were grown in a greenhouse at temperature of 20-22°C. The highly susceptible species Chico for *Pst0* and Ideal for *Xcv* were used as control. The symptoms of the disease were recorded on the 4-5th day. The assessment was made according to CHAMBERS-MERRIMAN's scale (1975) for *Pst* and to SOTIROVA-BELEVA's scale (1975) for *Xcv*. The plants which had no visible symptoms were defined as resistant.

RESULTS AND DISCUSSION

Resistance to *Pst*R0 was observed in hybrids derived from *L. pimpinellifolium*, *L. racemigerum*, but this is the first time that resistance is observed in crossings containing *L. humboldttii* (Table 1).

Table 1 : Reaction of tomato lines to race O of Pst and tomato and pepper-tomato pathotypes of Xcv

Line number	Genetic origin	Average spot number/plant		
		Pst R0	XcvT	XcvPT
10	83602029 x *L. minutum*		9.13	
12	*L. pimpinellifolium* x 86E2208	4.07		
22	86E2208 x 836020030	7.73		
30	83602030 x 83602029			8.30
34	83602030 x 83602027		9.74	
38-1	*L. racemigerum* x 86E2208	8.11		
50	86E2208 x *L. humboldtii*	8.50		
56	83602029 x Spectar	7.75		
11	86E2208 x *L. pimpinellifolium*	4.76	6.40	
21	86E2208 x 83602027		9.50	7.95
28-2	83602027 x 83602029		6.70	6.30
59	86E2208 x Spectar		5.90	7.93
77	Spectar x 83602027		7.25	9.46
82	Mercury x 83603043	5.70	9.75	8.32
St	Chico	89.56	98.57	120.12
St	Pepper			19.85

Of particular interest are the resistant hybrids, which were obtained by combination of the local sample 86E2208. They are large fruited and resistant to all forms of injury from *Alternaria solani*.

The large fruited hybrids containing germ plasm of the variety Spectar were found resistant to the two strains of *Xcv* (*XcvT, XcvPT*). Crossings involving Mercury were found resistant to both pathogens *Pst* and *Xcv*. The high variation values about sensitivity, as well as the great percentage of plants, manifesting a hypersensitive reaction, provide a possibility for screening of the more resistant lines to the investigated pathogens in the following generations.

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Efficacy of copper chelate to control bacterial dieback of peaches (*Pseudomonas syringae* pv. *persicae*)

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ABSTRACT

Two new chemicals were checked for their efficacy to control the bacterial dieback which is occuring on peaches in the SE France and which is caused by *Pseudomonas syringae* pv. *persicae* (*P.s.p.*).

Experiments were performed either on young plants in an environment controlled chamber (Exp1) or in an orchard located in the area where the disease develops (Exp 2).

Bactifolistop and Pseudocid, both containing copper chelated by EDTA were compared to an antibiotic (Kasumin®) and to a copper salt (Bordeaux Mixture) as references, in their ability to reduce the epiphytic populations of *P.s.p.* (Exp 1 and 2) and correlatively the incidence of the disease (Exp 2).

Copper chelate, whatever the formulation, appeared to be as active as the antibiotic reference in reducing the epiphytic populations of *P.s.p.*. Moreover, it made the leaf fall period as short as 12 days so that only two sprayings instead of four were needed to control efficiently the bacterial dieback of peaches in orchard.

Key words : bacterial disease, peach, Pseudomonas, control .

INTRODUCTION

Pseudomonas syringae pv. *persicae* (*P.s.p.*), the causal agent of the bacterial dieback of peaches in France (VIGOUROUX, 1970 ; PRUNIER *et al,* 1970) colonizes as an epiphyte the surface of aerial parts of its host (GARDAN *et al*,

1972). Abundant epiphytic populations during autumn were shown to be responsible for the initiation of infection through leaf scars (LUISETTI *et al*, 1973 ; PRUNIER *et al*, 1973). A relationship was demonstrated between the autumnal level of epiphytic populations of *P.s.p.* and the incidence of the disease next spring but only when the environmental conditions were favourable (LUISETTI *et al*, 1973, 1984 ; VIGOUROUX, 1979).

Good control of the disease can be achieved by means of 3-4 sprayings of any bactericide during leaf fall to lower the epiphytic inoculum (PRUNIER *et al*, 1974 ; GAIGNARD *et al*, 1976). Copper salts (hydroxide, oxychloride and sulphate) and flumequine (Firestop®, 3 M Santé, Pithiviers, 45, France) are the only chemicals actually available (BRISSET *et al*, 1991).

Two new chemicals were tested during the last two years to determine their efficacy in the control of the bacterial dieback.

MATERIALS AND METHODS

Two types of experiments were performed according to the scheme defined by GAIGNARD *et al*, 1973, (i) on young peach seedlings grown in an environment controlled chamber (for Pseudocid only) (ii) in experimental orchard during autumn (for both Pseudocid and Bactifolistop).

Bacterial strain

Strain INRA M24-Sm^r of *P.s.p.*, isolated from peach was used. Calibrated suspensions (10^8 cells ml^{-1}) were prepared from 3-4 days old cultures grown on LPGA and incubated at 16°C.

Chemicals

Bactifolistop (SIFO, Pont de l'Arche, 27, France) and Pseudocid (Guyon, Goussainville, 93, France) are both compounds derived from EDTA ; they contain respectively 9,5 and 6,4 ‰ of copper and were used at the concentration of 1,25 ‰ copper. They were compared to three reference bactericides, Kasumin® (Hokko Chemical Industry Co Ltd, Tokyo, Japan) used at the concentration of 0,5 ‰ Kasugamycin, Firestop® at the concentration of 0,3 ‰ flumequine and Bordeaux mixture (Atochem, St Marcel, 27, France) at the concentration of 1,25 ‰ Copper.

Experiment in environment controlled chamber

Bacterial suspension was sprayed on young GF 305 peach seedlings untill run off. Plants were then kept for 7 days in an environment controlled chamber

(24/19°C ; 16/8 hrs ; 3000 lux) to favour bacterial colonization of leaves surface. Chemicals at the required concentration were then sprayed untill run off and plants were placed again in an environment controlled chamber. Populations for each treatment were assessed by recovering bacterial cells from 30 leaves sampled just after chemicals spraying and then 1, 2, 4, 7 and 10 days after.

In experimental orchard

Bacterial suspension prepared as previously mentionned was sprayed at mid-september on 3 years old peaches cv. Early O'Henry grown in experimental orchard in the SE of France where the disease is commonly occuring.

After a 7 days period for bacterial establishment on the trees, the chemicals were sprayed and then sprayed again twice at 5 and 10 days interval. Bacterial populations for each treatment were assessed on 60 leaves sampled just before each chemical spraying and 4 days after the second and the last one.

Disease incidence was assessed the next february on the treated and untreated trees.

RESULTS

1) in environment controlled chamber (table 1 - fig 1)

The population of *P.s.p.* on the control increases within the two first days then remains stable untill the end of the experiment.

Dates of Sampling	Treatments			
	Control	Kasumin	Bordeaux Mixture	Pseudocid
0	3,00	4,24	4,18	4,30
1 day	3,69	1,70	4,08	4,11
2 d	3,99	2,18	3,81	2,18
4 d	3,77	2,65	3,69	3,20
7 d	3,66	1,70	3,46	3,37
10 d	3,70	1,70	3,30	3,00

Table 1 : Dynamics of *P.s. persicae* populations (expressed as the logarithm of the number of bacteria per leaf) on peach seedlings according to the chemical sprayed (environment controlled chamber, 24-19°C, 16/8 hrs, 3000 lux).

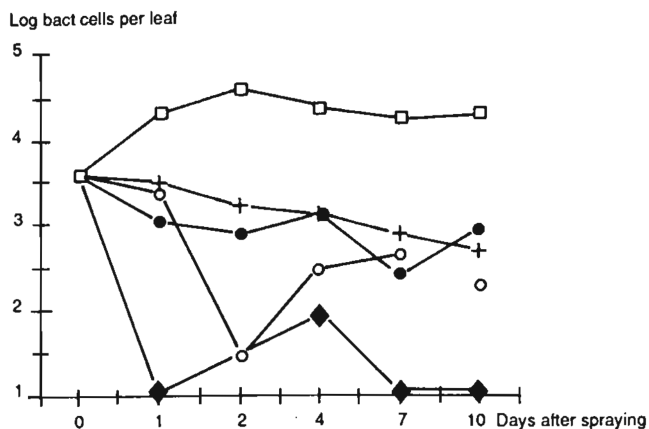

Figure 1 : Effect of Pseudocid and Bactifolistop on the dynamics of epiphytic populations of P.s.p. compared to reference chemicals (experiment in environment controlled chamber).

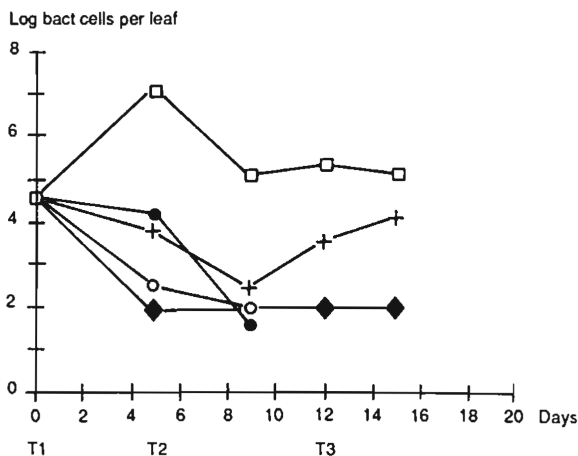

Figure 2 : Effect of Pseudocid and Bactifolistop on the dynamics of epiphytic populations of P.s.p. compared to reference chemicals. Three treatments were applied (T1, T2 and T3) for the reference and two only for the tested chemicals (T1 and T2) (Orchard experiment).

Values used for the graphes were issued from a transformation of the data shown in the corresponding tables in order to start with the same population level.

—□— Témoin
—+— T. Antibio.
—○— T. Cuivre
—●— Pseudocid
—◆— Bactifolistop

In the case of the seedlings sprayed with Kasumin the population drops down within the first day and then remains at a low level despite some variations. For the seedlings treated with Pseudocid, the population also drops down but within two days and then increases against a quite slow rate, reaching however a level significantly lower than that estimated for the control or for the Bordeaux Mixture.

2) in experimental orchard (table 2, fig. 2)

Within the five days separating the first two chemicals sprayings, the population on the control trees increases strongly (+ 2,5 as expressed in logarithm of the number of bacteria per leaf). During the same period the populations on the trees treated with Kasumin, Pseudocid, Bactifolistop and Bordeaux Mixture decrease but more with Kasumin than for the other chemicals.

After the second chemicals spraying the populations on the trees treated with either Pseudocid or Bactifolistop decrease again to reach a rather low level similar to that observed with Kasumin.

Dates of sampling	Treatments				
	Control	Kasumin	Bordeaux Mixture	Pseudocid	Bactifolistop
Before the first spraying	3,44	4,42	4,66	4,48	4,71
Before the second spraying (5 days after the first)	5,90	1,70	3,83	2,30	4,20
4 days after the second spraying	3,84	1,70	2,40	1,70	1,70
Before the third spraying (6 days after the second)	4,06	1,70	3,47	NR	NR
4 days after the third spraying	3,84	1,70	4,05	NR	NR

NR : No result - no more leaves on the trees

Table 2 : Dynamics of *P.s. persicae* populations (expressed as the logarithm of the number of bacteria per leaf) during the leaf fall according to the chemical

sprayed (experimental orchard).

Besides this antibacterial effect, both compounds cause an early entire leaf fall so that there are no more leaves on the treated trees 6 days after the second spraying.

Looking at the dynamics of the populations on the control trees, we observed they remain at a quite high level despite some variations. The same applies to the population on the trees treated with Bordeaux Mixture wich is estimated at about 10^4 at the end of the leaf fall. For Kasumin which is known to be very active against Pseudomonads, the population remains at a very low level till the end of the fall.

The next february only a few necroses were observed in the experimental orchard, 13 were found on the four untreated trees. No symptom was recorded on the treated trees.

DISCUSSION

Pseudocid and Bactifolistop are two different copper compounds derived from EDTA. They have demonstrated in two experiments their ability to significantly reduce *P.s.p.* populations on peach trees. Their bactericidal efficacy appears to be similar to that of Kasumin, an antibiotic previously proved to be a good bactericide against *Pseudomonas syringae* and better than that of the classical copper sulphate.

In field experiment, Pseudocid and Bactifolistop have led to an anticipated defoliation, causing a significant shortening of the leaf fall period. Moreover, they were able to protect the treated trees against the disease (no necrosis recorded on the 4 treated trees and 13 necroses on the untreated), eventhough the disease has not severely spread during the experimental period.

CONCLUSION

Pseudocid and Bactifolistop can be recommended to control bacterial dieback of peaches. Their efficacy to reduce the bacterial inoculum in autumn and to protect peaches against the disease appears undoubtfull. Moreover, since they cause a significant shortening of the leaf fall period (10-12 days instead of 30-35 days for untreated trees), two, instead of four sprayings for Firestop or Bordeaux Mixture, are required to control the bacterial dieback.

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Screening for resistance to pea bacterial blight (*Pseudomonas syringae* pv. *pisi*)

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ABSTRACT

Earlier studies on the genetics of the interaction of pv. *pisi* races and *Pisum* cultivars demonstrated that strong resistance was the result of a gene-for-gene interaction. Resistance (R) genes in the host interact with avirulence (A) genes in the pathogen to give a resistant response. Races were defined by their reactions on a differential series of eight cultivars carrying five R genes in different combinations.

In a further development of this study, a test array of *Pisum* germplasm from the John Innes collection was screened for resistance to races 1-6, containing all known avirulence genes. Tests were made on 60 plants of each accession (10 plants per race). The majority of accessions tested so far have reaction patterns equivalent to the known differentials. Some of the accessions were genetic mixtures and some contained R genes in combinations not previously encountered (e.g. R1 and R4 separately). Several new categories were also identified including a possible new gene (R6?) found in a Chinese accession. Another new source of resistance was found in accessions of *Pisum abyssinicum*. This shows resistance to all races including race 6 (the super race) and it is possible that this may be due to a form of race non specific resistance.

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