Margarita Elvira-Recuenco

SUSTAINABLE CONTROL OF PEA BACTERIAL BLIGHT

Approaches for durable genetic resistance and biocontrol by endophytic bacteria

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The research described in this thesis (April 1995-October 2000) was conducted at Plant Research International, Wageningen, The Netherlands and at Horticulture Research International, Wellesbourne, UK. In 1999 part of the research was also done at John Innes Centre, Norwich, UK.

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SUSTAINABLE CONTROL OF PEA BACTERIAL BLIGHT

Approaches for durable genetic resistance and biocontrol by endophytic bacteria

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STELLINGEN/PROPOSITIONS

1. The combination of race specific resistance and race non-specific resistance is the optimal genetic background for a potentially durable resistance to pea bacterial blight.
   This thesis

2. A differential expression of resistance to pea bacterial blight occurs in stem, leaf and pod for both race specific and race non-specific resistance.
   This thesis

3. Indigenous populations of endophytic bacteria in pea are affected by the plant genotype.
   This thesis

4. For a practical application in commercial pea growing of bacterial endophytes as biocontrol agents, it is necessary to be aware of the influence of environmental factors on endophytic colonization.
   This thesis

5. Genetic resistance and biocontrol by endophytic bacteria are complementary measures and potentially additive for a durable and sustainable control of pea bacterial blight.
   This thesis

6. The induction *in vitro* of an L-form (cell wall-less) bacterium is pathovar specific.
   This thesis

7. Error is all around us and creeps in at the least opportunity. Every method is imperfect.
   Charles Nicolle (1866-1936)

8. The way to grasp the integrity of the species can only be found in a synthesis of taxonomy, differentiating geography, genetics and cytology.
   N.I. Vavilov. Centers of origin of cultivated plants. Tr. po prikl. bot. I selek. (Papers on applied botany and plant breeding), vol. 16, no. 2, 1926.

9. La inspiración existe, pero tiene que encontrarte trabajando.
Inspiration exists, but it must find you working.
Pablo Ruiz Picasso (1881-1973)

10. No son buenos los extremos aunque sea en la virtud.
Extremes are not good even in virtue.
Santa Teresa de Avila (1515-1582)

11. 'Parece, Sancho, que no hay refrán que no sea verdadero, porque todos son sentencias sacadas de la mesma experiencia, madre de las ciencias todas'.
'It seems, Sancho, that there is no saying that is not true, since all sayings are statements derived from experience which is mother of all sciences'.
Don Quijote de La Mancha (1605-1615), Miguel de Cervantes Saavedra

12. 'There were once five peas in one shell, they were green, the shell was green, and so they believed that the whole world must be green also, which was a very natural conclusion'.
The Pea Blossom, 1853, Hans Christian Andersen

Margarita Elvira-Recuenco
'Sustainable control of pea bacterial blight: approaches for durable genetic resistance and biocontrol by endophytic bacteria'
Wageningen, 6 October, 2000

Preface

I have spent five years and six months working on this PhD study (16 April 1995-6 October 2000) in The Netherlands and England. What I cannot recall precisely is how long I have stayed in each country, it has been The Netherlands-England, England-The Netherlands during these five years; it is obvious my choice was not based on the need for a good climate! But it was the right choice.

The Spanish institution INIA, with its financial support, gave me the opportunity to do this study abroad, and I am very grateful to them, also for making an exception in allowing these studies to be carried out in two countries; a special mention goes to Luis Ayerbe, Vicente Reus and Carmen Alvarez. The project had a clear aim: measures for the control of pea bacterial blight. Nevertheless, two very distinct approaches had to be undertaken, which implied not only a degree of difficulty but also a great challenge. Indeed the benefits from this broad research have compensated for the difficulties, for which the contribution of my supervisors, Jim van Vuurde in The Netherlands and John Taylor in England, has been essential. I have learned many things from them about Plant Sciences and also about the art of science; we have shared many points of view and there has been an understanding and tolerance for those we did not share. It has been an intensive learning period under their guidance but also with freedom to undertake my own initiatives. Thanks are due also to my supervisor from the University, Mike Jeger, for following up the project, particularly during the last year, at a distance, and for encouraging me to write during my PhD study.

In 1999, two-month's work was conducted at John Innes Centre, England. The outcome of this short period was relevant. I deeply thank Noel Ellis for his supervision and support in elucidating the complex genetics of the breeding programme and for the pleasant time I spent in his lab.

Thanks are also due to Joe Kloepper, Auburn University, Alabama. During my visit to his lab I learned not only the conceptual basis of Induced Systemic Resistance but also the importance of the field screening in biocontrol.

I would like to thank very much all who contributed to this thesis in one way or another: help in the practical work in the lab, glasshouse, field, computing, library and for their advice, support and friendship. I have been very fortunate with the people I have met and it is not my purpose here to list everyone. Some of them have been acknowledged in the Chapters of this thesis. I would just like to mention a few names however: my colleagues at 'Bacteriologie', Wageningen, for their kindness, great fun in the lab, comradeship, speaking English to me, and above all, for making me feel a part of the group and never a stranger. This includes Ineke, José v. Beckhoven,

Maudie, Jan, Petra, Pieter, Henry, Sylvia, José Rasing and Els. Also to our foreign guests, Flori, Ednar, Janni, Veena.....,and trainees. Ineke, thanks for the good times we spent and of course for our traditional Chinese lunch. Jan, your advice and interesting discussions were an inestimable help for me.

My colleagues at Plant Pathology, Wellesbourne, for their kindness, Josie and Sara, it was great to share the office with you! Jan, Barbara and Pat, thanks a lot for your excellent work with the breeding material, and Joana, Paul and Steve, for your scientific advice.

A special word of thanks goes to my Dutch and English friends who helped me to know and love the Netherlands and England, and to my 'foreign' friends who showed me so much about different cultures, ways of living and supported me in overcoming difficulties when I felt homesick.

And at the end of this preface I return to the beginning...I mean, to my roots, y esto no es en inglés.... A mis amigos españoles, algunos fuera de España. A mis amigos de Móstoles, de toda la vida, con los que la amistad sigue siendo la misma después de estos muchos años de ausencia. Un recuerdo muy especial va para uno de ellos, Petri, que se fue de nuestro lado el año pasado. A mi familia de Cuenca, mis padres y mi hermana, muchas gracias mamá, papá y Yoli, sin vosotros esta tesis no hubiera sido posible. A mamá y Yoli decirlas que no puedo imaginar haber tenido un apoyo mejor que el suyo.

> I know not what I may appear to the world, but to myself I appear to have been only like a boy playing on the sea-shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.
>
> *Isaac Newton (1642-1727)*

Contents

Author's abstract

Elvira-Recuenco, M. 2000. Sustainable control of pea bacterial blight: Approaches for durable genetic resistance and biocontrol by endophytic bacteria. PhD Thesis, Wageningen University, The Netherlands, 200 pp., English, Dutch and Spanish summary.

Pea bacterial blight (*Pseudomonas syringae* pv. *pisi*) occurs worldwide and can cause severe damage under cool and wet conditions particularly at the seedling stage in winter- sown crops. Seven *Ps. syr.* pv. *pisi* races are currently recognized. There are no resistant cultivars to race 6, which is becoming increasingly important. Current disease control measures include disease avoidance through seed testing and the deployment of resistant cultivars with race specific resistance gene(s). In the present study two novel control measures were investigated with the potential for integration to give a durable and sustainable disease control. The first was breeding for resistance based on race non-specific resistance present in *Pisum abyssinicum*, which confers resistance to all races, including race 6. Its mode of inheritance was investigated through a crossing programme with *Pisum sativum* cultivars. Resistance was controlled by a major recessive gene and a number of modifiers. Progenies of crosses between resistant F5 populations and commercial cultivars are now available. Molecular markers for race non-specific resistance based on a pea retrotransposon marker system were developed. It is suggested that the combination of race specific and race non-specific resistance provides the optimal genetic background for the maximum expression of resistance to all races of the pathogen in all plant parts and under field conditions. The second measure was biological control by endophytic bacteria. Studies on the ecology of endophytic bacteria in pea and identification of efficient indigenous colonizers for potential application in biocontrol have been made. Endophytic population levels were in the range 10^3-10^6 CFU/g fresh tissue in roots and stems. There was a predominance of Gram-negative bacteria, particularly *Pseudomonas* sp. and *Pantoea agglomerans*. *Arthrobacter* sp. and *Curtobacterium* sp. were the main Gram-positive bacteria. Factors such as soil type, plant genotype and crop growth stage may significantly influence the diversity and population levels of endophytic bacteria. Future research should focus on the combination and testing of elite breeding lines with selections of disease suppressive endophytic isolates under a variety of field conditions in order to obtain an efficient and durable performance in commercial agriculture.

Key-words: bacterial blight, biological control, biodiversity, endophytic bacteria, L-form, pea, PDR1 retrotransposon, *Pisum sativum*, *Pisum abyssinicum*, *Pseudomonas syringae* pv. *pisi*, race specific resistance, race non-specific resistance, Spanish landraces.

Chapter 1

General Introduction

Pea bacterial blight: pathology and prospects for durable control

Pea cultivation can be traced to the Neolithic period (Zohary and Hopf, 1973). Most cultivated pea types are closely related to wild ecotypes found in the Middle East. Secondary centres of diversity have been identified in the highlands of South Central Asia and Ethiopia (Ellis *et al.*, 1998). Domestication involved evolution from taller and more rambling growth habit, tough seed coat, dehiscing pods and smaller seeds present in the wild types.

The number of species within the genus *Pisum* has been controversial. Davis (1970) reported at most two species (*P. sativum* and *P. fulvum*) while Ellis *et al.* (1998) reported that the genus *Pisum* has three main groups: *P. fulvum*, *P. abyssinicum* and other *Pisum* spp. (*P. elatius*, the highland Asiatic *P. sativum* and all the rest including modern cultivars). The tremendous variability in the present pea gene-pool reflects its early domestication and subsequent widespread distribution.

Mendel's work on hybridization with peas published in 1865 was the foundation of genetic science. However, it was not until 1901 that his work was rediscovered and its importance recognized (Bateson, 1901). The pea has been an important model plant for several generations of plant physiologists, biochemists and geneticists (Davis, 1993).

Peas are grown worldwide, but because of sensitivity to extremes of climate, especially high temperatures, are largely confined to temperate regions and the higher altitudes or cooler seasons of warmer regions. They require well-drained soils and are especially sensitive to water and temperature stress during germination and flowering. Pea crops are grown for a number of specialist purposes: (1) Vining or garden peas, where plants are harvested for their tender green immature seeds, with a high sugar content, and used for human consumption either fresh, frozen or canned; (2) Mangetout and sugar snap peas, where plants are harvested for human consumption of their fresh immature pods; (3) Dried or combining peas, where the crop is harvested as matured dried seeds which are used for human consumption directly after rehydration or after preservation by rehydration and canning, they may also be used in animal feed and as a raw material for protein and starch extraction; (4) Forage peas, where the whole plant is harvested at the flat pod stage and may be ensiled or dried and used as hay; and (5) Green manure crop.

Total world production of dry peas in 1998 was 12,2 million metric tones, the fourth most important legume (FAO, 1998). The main areas of production are USA, Canada, India, China, Russian Fed., France, Germany, Denmark and UK. During the 1980s there was a dramatic expansion in the area of dry peas grown in the EC, particularly in France, Denmark and the UK, due to the EC policy to increase cultivated surface and encourage research: from 111,000 ha with yields of 3,300 kg/ha

in 1979 to 900,000 ha and yields of 4,500 Kg/ha in 1991. In Spain, increase on the cultivated surface did not occur until the 1990s: from 9,000 ha in 1991 to 70,500 in 1994. The average yield in the EC in 1998 (4,400 kg/ha) is much greater than the world average (1,900 kg/ha). However, yields in Spain are still very low (1,300 kg/ha).

Pea is an important protein source for use in compounded animal feed which could further contribute to a reduction in the European dependency on soya imports. Sustainable agriculture practices are increasingly demanded and grain legumes, including peas, with their ability to yield well in the absence of added nitrogen fertilizer, through symbiotic nitrogen fixation with *Rhizobium*, provide a valuable component in crop rotation systems.

There is an unquestioned need to improve the level of protection to the main fungal, bacterial and viral diseases (Hagedorn, 1985) in order to increase the yield and quality of the pea crop. Hagedorn (1985) reported as major diseases in the pea crop: Pythium damping-off caused by *Pythium ultimum* Trow and/or other *Pythium* species; 'Ascochyta' diseases which include Ascochyta leaf and pod spot caused by *Ascochyta pisi* Lib., Ascochyta blight by *Mycosphaerella pinodes* (Berk and Blox) and Ascochyta foot rot by *Phoma medicaginis* var. *pinodella* (Jones) Boerema (ex *Ascochyta pinodella* Jones); near-wilt by *Fusarium oxysporum f.* sp. *pisi* race 2; Aphanomyces root rot by *Aphanomyces euteiches* Drechs and Fusarium root rot by *Fusarium solani* f. sp. *pisi* (F.R. Jones) Snyd.&Hans.

Bacterial blight (*Pseudomonas syringae* pv. *pisi*) is considered to be of moderate importance (Hagedorn, 1985), but under cool and wet conditions, at first stages of development, severe losses may occur. The lack of resistant cultivars to race 6 of *P. syr.* pv. *pisi* and the increasing incidence of this race may result in a greater importance of this disease in the near future. It is considered one of the three most damaging diseases in the Spanish pea crop together with *Ascochyta pisi* and *Botrytis cinerea* (Laguna *et al.*, 1997).

1. DESCRIPTION OF PEA BACTERIAL BLIGHT

1.1. The causal organism

Pea bacterial blight caused by *Pseudomonas syringae* pv. *pisi* was first recorded in Colorado (Sackett, 1916). It is a Gram-negative, non-spore forming rod (*ca.* 0.7 x 2-3 μ), chemo-organotrophic, obligative aerobe, motile by one to five polar flagella. Optimum growth occurs at 26-28°C and pH 6.5-7.5. Most strains produce a yellowish-green diffusible pigment on King's B medium which fluoresces blue under ultra violet

light.

It has the typical characters of a LOPAT group 1a green fluorescent Pseudomonad (Lelliot *et al.*, 1966): positive levan production on 5% sucrose nutrient agar (white mucoid colonies), negative oxidase, negative pectolytic activity on potato slices, negative arginine dihydrolase activity and positive hypersensitive reaction in tobacco.

1.2. Disease symptoms and potential losses

Pea bacterial blight may affect all aboveground plant parts. Under cool and wet conditions, favourable for the spread of the disease, lesions are initially discrete, shiny and water-soaked, on stipules lesions may be fan shaped, with age lesions become darker and finally necrotic. In warm dry weather, less favourable for the disease, lesion development will be arrested and water-soaking may be less obvious and lesions smaller.

Pathogen invasion of the intercellular spaces causes the plant cells to leak their contents causing water congestion of the tissues and hence the initial water-soaked appearance of the lesions. The bacteria then feed on the leaked nutrients and multiply in the intracellular spaces (Hunter, 1996). In the case of a resistant response, the so-called hypersensitive reaction is expressed as the rapid collapse and browning of invaded tissues in association with the accumulation of phytoalexins and the inhibition of bacterial multiplication (Cruishank and Perrin, 1961).

When peas are infected at the seedling stage, the entire crop may be lost. Irregular maturity results when the growing tip is killed at later stages, defoliation, blasting of blossoms and pods and unsightly pods can lower yield, quality and the value of the crop (Hagedorn, 1989).

Ps. syr. pv. *syringae* and *Ps. viridiflava* can be found in association with *Ps. syringae* pv. *pisi* (Taylor and Dye, 1972). Infections in the field by *Ps. syr.* pv. *pisi* and *Ps. syr.* pv. *syringae* are generally indistinguishable. The organisms can be distinguished by host tests in the glasshouse as well as serological (agglutination with specific antisera) and nutrtional tests (homoserine utilization).

Occurrence of pea bacterial blight has been reported in all continents (Anon., 1971; Hunter and Cigna, 1981; Taylor, 1986; Hollaway and Bretag, 1995*a*). The first occurrence of the disease in Europe was described in The Netherlands (1924), and subsequent occurrences were described in most other European countries. Although the disease was known to be present in Spain (A. Ramos-Monreal, personal communication), when the present study was initiated there was no published record on its occurrence in Spain.

1.3. Disease transmission

1.3.1. Seed borne transmission

Pea bacterial blight is a seed borne disease (Skoric, 1927). Epidemics may be initiated from seed infection levels of 0.1% or less. The expected transmission ratio seed:seedling is approximately 10:1 under normal field condition (Taylor, 1982). Skoric (1927) reported overwintering of *Ps. syr.* pv. *pisi* as a dry bacterial film on the surface of the seed and in the seed coat. Hagedorn (1989) reported that the bacterium was carried by the seed both externally and internally and could persist for at least three years, it did not appear to penetrate the embryo or cotyledon and the most common primary sites of seed-induced infection were the lower stipules.

1.3.2. Secondary spread in the field

Sackett (1916) reported penetration through wounds and stomata into the stem and leaves and further spread into the underlying parenchyma and that the infection did not appear to spread into the pith and vascular bundles. He observed a gradual wilting of plants but not a sudden collapse. However, Skoric (1927) observed that the bacteria enter parenchyma cells of the cortex and the pith breaking down cell walls and vessels by high pressure of bacterial slime and by chemical action, and then may enter into the vascular bundles, with consequent wilting of leaflets and occasionally of the whole plant (Skoric, 1927).

Secondary infection and spreading of the disease is favoured by any activity which can disseminate bacteria and cause wounding: rain and wind damage (Stead and Pemberton, 1987), farm machinery, people, insects and birds (Roberts, 1991), irrigation, frost and hail damage (Young and Dye, 1970; Boelema, 1972). Mansfield *et al.* (1997) found that disease severity was greater in winter-sown than spring-sown peas and that yield reduction was strongly correlated with disease severity.

The soil is not a primary source of inoculum and the pathogen as a free living organism is unlikely to survive from one season to the next. However, infected plant debris in soil is a potent source of inoculum and crop rotations which include two seasons free of field peas should be considered as part of a strategy to control bacterial blight (Hollaway and Bretag, 1997). Weeds collected in naturally infected pea fields often harboured the pathogen but with levels smaller than those observed on peas (Grondeau *et al.*, 1996). Among alternative hosts reported are sweet pea (*Lathyrus odoratus*), red clover (*Trifolium pratense*) and soybean (*Glycine max*) (Hagedorn, 1989). *Ps. syr.* pv. *pisi* has an epiphytic resident phase and its development on pea is

pathogen race specific (Grondeau *et al.*, 1996). It is not known whether the same genes for pathogenicity are also involved in the race-specificity of epiphytic development.

Soil moisture contents influence transmission of infection from seed to seedling (Skoric, 1927; Roberts, 1992; Hollaway *et al.*, 1996), but not temperature (Roberts, 1992). The influence of soil moisture suggests that the embryo itself is not actually infected and that infection takes place during germination and emergence. By sowing later in the year the likelihood of a drier seedbed is increased which would result in a lower incidence of disease transmission, seed to seedling. Later sowing may give a yield penalty but may be worthwhile for seed crops (Roberts, 1992).

1.4. Race occurrence

Two races of *Ps. syr.* pv. *pisi* were defined for the first time by Taylor (1972) on the basis of different reactions on two pea cultivars, Early Onward and Partridge. A further four race types were later identified on the basis of interactions with an expanded set of nine cultivars (Taylor *et al.*, 1989; Bevan *et al.*, 1995). Race 6 was originally found as a spontaneous mutation from race 3 in a laboratory culture but has since been found in naturally infected pea crops. Race 6 is unique in its ability to cause disease in all cultivars tested (Taylor *et al.*, 1989) and at present there are no cultivars known to be resistant to race 6.

Race typing of a collection of 146 isolates from UK and overseas showed that race 2 was predominant (83% of all UK isolates) (Taylor *et al.*, 1989). In a study made in the UK in seed stocks from 1987 to 1994 (Reeves *et al.*, 1996), race 2 was most frequently isolated (65% to 92%), races 1, 3, 4 and 5 occurred infrequently and there was an increase in the incidence of race 6, representing 26% of the infected samples in 1994. Schmit (1991) in a study in France, also reported the predominance of race 2 (52% of the isolates), followed by race 6 (36%) and 4 (12%). The race frequency spectrum in Australia (Hollaway and Bretag, 1995*b*) is quite different from Europe: race 3 represented 64% of the isolates tested, race 6 (31%) and race 2 (5%).

1.5. Measures for the control of pea bacterial blight

Primary infection can be prevented by the use of disease-free seed and therefore disease avoidance through seed testing is a major control measure of pea bacterial blight.

Measures to avoid spreading of the disease once this has been introduced in the

field are: (1) Use clean farm equipment, (2) Avoid sprinkler irrigation, particularly in seed crops, (3) Plough in infected crops immediately after harvest and disinfect all equipment, (4) Do not grow peas in fields for at least one season after an infected crop and (5) Destroy all volunteer peas before resowing.

The use of resistant cultivars is also a key-measure for the control of the disease and race specific resistance genes are present in many cultivars. It would be optimal to use cultivars that are resistant to all races of the pathogen, however, all cultivars tested so far are susceptible to race 6. Biological control of the disease constitutes an elusive control measure.

2. BREEDING FOR RESISTANCE TO PEA BACTERIAL BLIGHT

Pea is diploid with seven chromosome pairs. It is self-fertilizing and natural outcrossing has been estimated to be less than 1% (Gritton, 1980).

2.1. Race specific resistance

2.1.1. The gene-for-gene model

The genetic analysis of six races of *Ps. syr.* pv. *pisi* and a set of nine differential pea cultivars allowed a gene-for-gene model to be proposed based on five matching pairs of resistance genes (R-genes) in the host and avirulence genes in the pathogen (Taylor *et al.*, 1989). The model indicated one or more avirulence genes operating in each of the six known races with the exception of race 6, which was found to be compatible on all cultivars tested. Further genetic analyses of both the host and pathogen and the discovery of one new naturally occurring race subsequently led to the revision and refinement of the model in which the interaction of eight differential cultivars with seven races was based on six matching pairs of resistance genes in the host and avirulence genes in the pathogen (Table 1, Bevan *et al.*, 1995).

2.1.2. Frequency of race specific resistance genes

Resistance to races 1-5 was found to be widespread in a collection of *Pisum* sp. germplasm including 151 lines (commercial cultivars, breeding lines and wild types), with more than 75% showing resistance to one or more races, indicating the widespread presence of race specific R-genes. The predominant R-gene was R3 (56%) followed by R2 (38%) and R4 (11%) (Taylor *et al.*, 1989). In a later *Pisum* sp.

germplasm screening, part of a CEC programme, which included 231 accessions, R3 was also found to be the most common R-gene (J.D. Taylor, personal communication).

There seems to be an involvement of host genotype in the occurrence of specific races, which has been also reported in the soybean-*Ps. syr.* pv. *glycinea* interaction (Cross *et al.*, 1966). The most common commercial pea cultivars grown in Australia are susceptible to races 3 and 6 of *Ps. syr.* pv. *pisi*, where these races have a high incidence (Hollaway and Bretag, 1995*b*). The lower frequency of race 2 in Australia than in UK and France is most likely due to the presence of R2 in common cultivars in Australia. Many of the cultivars grown in UK and France lack R2 and are susceptible to race 2, likewise many Australian cultivars lack R3 and are susceptible to race 3. Schmit (1991) found that race 2 predominates in the North of France where there is intensive production of spring cultivars (most of them susceptible to race 2). Most of the strains identified as race 6 were found in the South of France were winter cultivars are grown (with frequent resistance to race 2).

Table 1. Gene-for-gene relationship between pea cultivars and races of *Pseudomonas syringae* pv. *pisi* (Bevan *et al.*, 1995)

							Race/avirulence genes						
							1	2	3	4	5	6	7
							1	.	.	.	.	.	.
							.	2	.	.	2	.	2
							3	.	3	.	.	.	3
							4	.	.	4	4	.	4
							.	.	.	.	(5)	.	.
	Resistance (R) genes						(6)	.	.	.	(6)	.	.
KelvedonWonder	.	.	.	.	.	.	+	+	+	+	+	+	+
Early Onward	.	2	.	.	.	.	+	-	+	+	-	+	-
Belinda	.	.	3	.	.	.	-	+	-	+	+	+	-
Hurst's Greenshaft	.	.	.	4	.	(6)	-	+	+	-	-	+	-
Partridge	.	.	3	4	.	.	-	+	-	-	-	+	-
Sleaford Triumph	.	2	.	4	(5)	.	-	-	+	-	-	+	-
Vinco	1	2	3	.	(5)	.	-	-	-	+	-	+	-
Fortune	.	2	3	4	.	.	-	-	-	-	-	+	-

+, Susceptible response; -, resistant response; genes in parentheses partly proven; ., gene absent

2.1.3. Genetic mapping of race specific resistance genes

Genetic analysis of the inheritance of resistance to *Ps. syr.* pv. *pisi* in pea provided evidence of linkage between the resistance genes R3 (*Ppi-3*) and R4 (*Ppi-4)* (Bevan *et al.*, 1995). Hunter (1996) confirmed the linkage between *Ppi-3* and *Ppi-4* and linkages to isoenzyme and morphological characters indicated that the linked loci could be associated with either linkage group I or VII. Mapping studies in two recombinant inbred populations placed R2 (*Ppi-2*) on linkage groupVII and located R1 (*Ppi-1*) on linkage group VI close to hilum colour allele *pi*.

2.1.4. Races/Avirulence genes

The avirulence gene A2 in race 2, was the first cloned avirulence gene (*avrPpiA*) involved in a gene-for-gene relationship in a *Ps. syringae* pathovar (Vivian *et al.*, 1989). This gene was found to alter the virulence of *Ps. syr.* pv. *phaseolicola* to bean and *Ps. syr.* pv. *maculicola* to *Arabidopsis* in a cultivar or ecotype specific manner (Dangl *et al.*, 1992). The activity of *avrPpiA* has therefore demonstrated the presence in bean and *Arabidopsis* of functional homologs of the R2 gene for resistance to *Ps. syr.* pv. *pisi* (Dangl *et al.*, 1992; Fillingham *et al.*, 1992).

The avirulence gene *avrPpiB* from *Ps. syr.* pv. *phaseolicola* races 3 and 4 was found to confer avirulence on *Ps. syr.* pv. *pisi* in all cultivars examined (Fillingham *et al.*, 1992; Vivian and Mansfield, 1993).

Wood *et al.* (1994) detected a gene in pea controlling nonhost resistance to *Ps. syr.* pv. *phaseolicola* (cloned DNA from a plasmid in *Ps. syr.* pv. *phaseolicola* conferred avirulence on *Ps. syr.* pv. pisi towards its host pea). Avirulence was determined by two loci which appeared to match a single dominant resistance gene in the pea cultivar Kelvedon Wonder the first gene for nonhost resistance to be identified in pea.

2.2. Race non-specific resistance

Race non-specific resistance to *Ps. syr.* pv. *pisi* was primarily detected during a *Pisum* sp. germplasm screening using a stem inoculation technique (Schmit *et al.*, 1993; Taylor *et al.*, 1994). All the accessions listed as *Pisum abyssinicum* were found to be resistant or partially resistant to all races of *Ps. syr.* pv. *pisi*, including race 6, for which there are no known commercial resistant cultivars. Sixteen of these accessions originated from Ethiopia and one from Yemen.

Pisum abyssinicum grows mainly in Northern Ethiopia at altitudes between 1800 to

3000 m. Ethiopia is one of the centres of diversity of cultivated plants (Vavilov, 1992). The climate is relatively wet during the vegetative period (usually spring) and harvests usually coincide with the dry period. Plants are tolerant to low temperatures, particularly during the early stage of plant growth. One of the characteristic properties of the Ethiopian pea is its cosmopolitan qualities: it can be successfully grown at he northern extremities of cultivation but succeeds also under dry arid steppe conditions. In addition to the mesophilic subgroups sown at the beginning of the rainy period, there are also xerophilic ones, sown at the end of the wet season and subject to the effects of drought (Vavilov, 1992).

Preliminary studies on the inheritance of race non-specific resistance derived from *P. abyssinicum* in a limited number of *P. sativum* x *P. abyssinicum* F2s pointed to a single recessive type of resistance (J.D. Taylor, personal communication).

3. BIOLOGICAL CONTROL OF PEA BACTERIAL BLIGHT BY ENDOPHYTIC BACTERIA

3.1. What are endophytic bacteria?

Research on bacteria residing in the internal tissues of non-symptomatic plants dates back to Pasteur (1876), who reported that grape juice was microorganisms-free when extracted aseptically. Papers published on the subject from 1876 to 1896 (reviewed by Smith, 1911) served only to inculcate the belief that healthy plant tissues were free of microorganisms and scientists reported the bacteria found within healthy plants as due to contaminants and not as natural colonizers. Since 1896 until 1950 authors reported on bacteria from internal plant tissue but with few exceptions no clear statements were established (Hollis, 1951).

Perotti (1926) first coined the term endophyte to describe the bacterial microflora other than *Rhizobium* spp. isolated from the root cortex of healthy plants. Several definitions of endophytic bacteria have been proposed since then (Kado, 1992; Quispel 1992; Beatti and Lindow, 1995). The definition given by Hallmann *et al.* (1997*a*) include those bacteria that can be isolated from surface-disinfested plant tissue or extracted from within the plant and do not visibly harm the plant. This definition does not include non-culturable and non-extractable endophytic bacteria and is inclusive of bacterial symbionts as *Rhizobium*. It is a functional and practical definition since it includes the broad spectrum of work being done on the presence, population dynamics and effect of non-pathogenic colonizers of internal plant tissue.

3.2. Isolation and examination of endophytic bacteria

The isolation procedure is a limiting factor when studying endophytic bacteria. An optimal isolation procedure should include only the complete internal bacterial population, however, in practice, this is unlikely to be achieved. The most common isolation technique has been surface-disinfestation and grinding. This technique might over or underestimate the bacterial endophytic populations due to several factors such as incomplete surface disinfestation, strong adsorption of bacterial cells to plant cell structures, and the penetration of the disinfestant into plant tissues (Hallmann *et al.*, 1997*a*). An alternative procedure used to overcome some of these constraints, is vacuum and pressure extraction to extract endophytic bacteria from xylem and intercellular spaces (Gardner *et al.*, 1982; Bell *et al.*, 1995). However, comparison of both techniques (Bell *et al.*, 1995; Mahaffee and Kloepper, 1997; Hallmann *et al.*, 1997*b*) indicates qualitative and quantitative differences, with the higher recovered numbers in the grinding technique most likely due to the fact that some bacteria clump together or tend to absorb to particles in the plant (Fisher *et al.*, 1992).

Plating on culture media is the simplest technique for monitoring endophytic populations. Non-culturable types will not be detected with this technique and the nutrient media will select for the fraction of the total population that can grow on the chosen medium (Bell *et al.*, 1995). Alternative techniques for examination of endophytes *in situ* are viable staining with 2,3,5-triphenyltetrazolium dichloride (Patriquin and Döbereiner, 1978; Bashan and Holguin, 1995), electron microscopy (Hinton and Bacon, 1995; Benhamou *et al.*, 1996*a*), and autoradiography (Sigee, 1990). For the study of specific endophytes, probe based systems as inmunological staining and quantification by ELISA (Levanony and Bashan, 1990; van Vuurde and Roozen, 1990; Mahaffee *et al.*, 1997), nucleic acid hybridization (McFadden, 1991; Hurek *et al.*, 1994) and by plating and denaturing gradient gel electroforesis (Garbeva *et al.*, 2000) proved to be valuable tools.

3.3. Ecology of endophytic bacteria

The main source of endophytic bacteria appears to be the rhizosphere soil (De Boer and Copeman, 1974; Sturz, 1995; Mahaffee and Kloepper, 1997; Hallmann *et al.*, 1997*a*). The importance of the phylloplane as a source of endophytic bacteria (Beattie and Lindow, 1995) has not been studied in so much detail as with the rhizosphere soil, however it might also play an important role in the case of endophytes specialized in the aerial part of the plant. Although endophytes have been detected within seeds (Mundt and Hinkle, 1976; McInroy and Kloepper, 1995*a*; Adams and Kloepper,

1996), the importance of seeds as source of endophytic bacteria remains controversial. Micropropagated material constitutes a particular source of endophytic bacteria (Leifert, 1989).

Entry into the plant tissue can be via stomata, lenticels, wounds induced by biotic or abiotic factors and areas of emergence of roots (Huang, 1986). Wounds that occur naturally as a result of plant growth are reported to be the main point of entry (Sprent and de Faria, 1988). However, during agricultural practice crops are also subjected to many processes that involve wounding. The mode of entry also depends on the bacterial species. Wounds and root emergence are not absolutely required and active penetration has been reported (Hurek *et al.*, 1994; Benhamou *et al.*, 1996*a*).

Once the bacteria have entered the plant, they either remain localized or spread in the plant. Systemic bacterial colonization seems to be affected by the plant part (Mahaffee *et al.*, 1997; Quadt-Hallmann *et al.*, 1997). Colonization of specific plant areas like xylem or root tip seems to be strain and species specific. The potential for seed transmission of applied endophytes is still questionable (Hallmann *et al.*, 1997*a*). In general, endophytic bacteria colonize intercellular spaces and xylem (Dong *et al.*, 1994; Hinton and Bacon, 1995) with only a few reports on intracellular colonization (Frommel *et al.*, 1991, Mahaffee *et al.*, 1997). Endophytic bacteria have been found in the vascular system but usually in relative low numbers (Ruppel *et al.*, 1992). It seems that spatially limited colonization in the vascular system is characteristic of endophytic bacteria and probably a major factor in differentiating them from plant pathogens (Braun, 1990; Vasse *et al.*, 1995). Research on the nutritional requirements of endophytic bacteria and availability of these nutrients for endophyte metabolism has been long neglected.

Population densities of indigenous endophytes found in different crops ranged generally from 10^3 to 10^6 CFU/g fresh weight (Hallmann *et al.*, 1997*a*). Introduced endophytes are usually found at levels of 10^3-10^5 CFU/g (Dong *et al.*, 1994). In both cases populations are usually higher in the roots and lower stem and decrease acropetally (Fisher *et al.*, 1992; Quadt-Hallmann and Kloepper, 1996). Gram-negative bacteria are usually predominant over Gram-positives representing 75-100%, of the total population (Gardner *et al.*, 1982; Gagné *et al.*, 1987; McInroy and Kloepper, 1995*b*; Bell *et al.*, 1995). Leifert *et al* (1989) reported a predominance of Gram-positives in micropropagated plants. The most common taxa belong to the Pseudomonaciaceae and Enterobacteriaceae families.

Biotic factors such as plant-associated microorganisms and plant-parasitic nematodes and insects may influence the bacterial endophytic population (Fisher *et al.*, 1992; Hallmann *et al.*, 1998). The influence of plant genotype on endophytic colonization is scarcely reported (Samish *et al.*, 1961; Bell *et al* 1995; Adams and Kloepper, 1998), although it is indeed an important factor in understanding plant-

endophyte interaction. Abiotic factors such as temperature, rainfall, soil properties and UV radiation that affect the colonization of bacteria in the rhizosphere and phylloplane, will also be likely to affect bacterial endophytic colonization. Differences in endophytic colonization from different soil types have been reported (Quadt-Hallmann and Kloepper 1996; Mahaffee and Kloepper, 1996, Hallmann *et al.*, 1999). This probably reflects the interaction of soil factors such as texture, pH and organic matter content.

3.4. Efects of endophytic bacteria

Endophytic bacteria may have deleterious, neutral or beneficial effects on their host to control plant pathogens (Chen *et al.*, 1995; Nowak *et al.*, 1995; Hinton and Bacon, 1995) or to promote plant growth (Van Peer and Shippers, 1989; Kloepper *et al.*, 1992; Nowak *et al.*, 1995).

In the 90s there has been a strong increase number of studies reporting disease reduction by the use of introduced endophytic bacteria. However, very few are yet reported to have practical large scale applications in commercial agriculture (Cook *et al.*, 1996). Introduced endophytic bacteria include those isolated from the crop being studied, from other crops, or soils or may be avirulent strains of the pathogen to be controlled. An avirulent cell wall-less strain of *Ps. syringae* pv. *phaseolicola* was reported to induce resistance to a virulent strain of the same pathogen in bean (*Phaseolus vulgaris*) (Amijee *et al.*, 1992).

Several reports have described variation among cultivars for disease supression (Vakili, 1992; King and Parke, 1993; Smith *et al.*, 1997), colonization of the host (Hebbar *et al.*, 1992), induction of resistance (Liu *et al.*, 1995) and induction of plant growth responses (Becker and Cook, 1988; Chanway *et al.*, 1988). Smith *et al.* (1999) found a genetic basis in tomato for interactions with the biocontrol agent (*Bacillus cereus*) against *Pythium torulosum*: they observed a significant variation among the lines of a recombinant inbred population of tomato on supression of *P. torulosum* by *B. cereus*, but also a significant phenotypic variation for resistance to *P. torulosum*. However, they found a negative correlation between resistance to *P. tolurosum* and disease supression by *B. cereus*.

3.5. Endophytic bacteria in pea

The natural incidence of endophytic bacteria in pea has only been investigated in ovules, seeds and pods (Samish *et al*, 1963; Mundt and Hinkle, 1976).

Studies on biological control of pea diseases have been focused exclusively on fungal diseases: control of Pythium-damping off with *Trichoderma* spp. (Harman *et al.*, 1980; Lifshitz *et al.*, 1986), *Ps. cepacia, Ps. fluorescens* (Parke *et al.*, 1991; King and Parke, 1993; Benhamou *et al.*, 1996*b*) and *Enterobacter cloacae* (Hadar *et al.*, 1993); *Fusasrium solani* f. sp. *pisi* with *Pseudomonas* sp. (Castejón-Muñoz and Oyarzun, 1995) and *Fusarium oxysporum* f. sp. *pisi* with *Bacillus pumilus* (Benhamou et al 1996*b*). Høflich and Ruppel (1994.) reported that inoculation with *Rhizobium* and an associative strain of the endophytic bacterium *Pantoea agglomerans* increased the growth and yield of pea.

4. STRATEGIES FOR THE DURABLE CONTROL OF PEA BACTERIAL BLIGHT

Breeding for resistance to *Ps. syr.* pv. *pisi* has been used as a measure of control of the disease, however, only race specific resistance genes had been introduced into the commercial cultivars. The increasing importance of race 6, for which there are no known resistant cultivars, together with the possible appearance of new races, made obvious the need to breed for race non-specific resistance. When the present study was initiated a new source of potential race non-specific resistance had recently been identified (*Pisum abyssinicum*) and was available. This resistance is a quantitative type that confers resistance to all known races of the pathogen. Since this resistance was thought to be of a different nature to race specific resistance, it was therefore particularly relevant to investigate its mode of inheritance. It was thought that a combination of race specific and non-specific resistance could be additive and provide an optimal genetic background for protection against pea bacterial blight.

An understanding of the biology of the pathogen in relation to: (1) frequency of race specific genes present in *Pisum* germplasm and race frequency, (2) differential responses to *Ps. syr.* pv. *pisi* in different plant parts and (3) performance of race non-specific resistance under field conditions, is also necessary to establish the guidelines for a successful breeding programme for resistance to pea bacterial blight with the prospect of long-term performance.

Biological control of pea bacterial blight could provide a measure for a durable control complementary with the use of resistant cultivars. Endophytic bacteria reside in internal plant tissues. These tissues may provide a more uniform and protective environment than plant surfaces where exposure to extreme environmental conditions and microbial competition are major factors limiting long-term bacterial survival. No studies have reported on the biological control of pea bacterial blight and studies on the indigenous endophytic bacterial population have been limited to ovules, seeds and

pods. Research should primarily focus on detection techniques for endophytic bacteria in pea, factors affecting endophytic bacterial colonization at the population level and taxa, and the building of an endophytic bacterial collection of indigenous types in pea to be further screened for biological control of *Ps. syr.* pv. *pisi*.

OUTLINE OF THIS THESIS

Chapter 2, the frequency of race specific resistance genes to pea bacterial blight in Spanish landraces is reported. Although *Ps. syr.* pv. *pisi* is a well established pathogen in Spain, this study represents the first published record of the occurrence of the disease in Spain.

Chapter 3, description of the inheritance of race non-specific resistance derived from *Pisum abyssinicum* through a crossing programme between two *Pisum sativum* cultivars (Kelvedon Wonder, susceptible to all races, and Fortune, resistant to all races except race 6) and two *P. abyssinicum* accessions (both resistant/partially resistant to all races but one of them with a higher rate of resistance). Additionally, the introduction of race non-specific resistance into commercial cultivars and the development of molecular markers to assist in the breeding programmes are described.

Chapter 4, the differential responses to *Ps. syr.* pv. *pisi* in different plant parts under glasshouse and field conditions and performance of race specific and non-specific resistance are reported.

Chapter 5, the development and evaluation of methods for the detection and isolation of endophytic bacteria in eleven pea cultivars are described and the differences in stem colonization of these cultivars are analyzed.

Chapter 6, the influence of soil type, plant genotype, growth stage of the crop and plant part on the population dynamics of endophytic bacteria in five *Pisum sativum* cultivars and one *Pisum abyssinicum* accession are reported.

Chapter 7, methodology for the induction of the L-form (cell wall-less) of *Ps. syr.* pv. *pisi* as a potential biocontrol agent of pathogenic *Ps. syr.* pv. *pisi*.

Chapter 8, General Discussion, the main findings are discussed and preliminary findings on the screening of endophytes for the control of pea bacterial blight are described.

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Chapter 2

Resistance to bacterial blight (*Pseudomonas syringae* pv. *pisi*) in Spanish pea (*Pisum sativum*) landraces

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ABSTRACT

Pea bacterial blight (*Pseudomonas syringae* pv. *pisi*) has long been known to be present in pea growing areas of Spain and to cause serious crop losses, although there is no published record of its occurrence. A collection of 16 isolates from a winter pea trial in Valladolid in 1991 which were shown in this study to be *Ps. syr* . pv. *pisi* races 4 and 6 would appear to be the first published record of the disease in Spain. This occurrence of races 4 and 6 is the same as reported for winter-sown peas in the South of France.

Ten *Pisum sativum* landraces from different geographical areas of Spain and considered to be representative of the traditional pea crop, were tested for resistance to seven races of *Ps. syr.* pv. *pisi*. Seedlings of each landrace were stem inoculated with the type strain of each race in a glasshouse. Resistance exhibited by the different landraces mainly conformed to those previously described in pea cultivars indicating various combinations of the main resistance genes: R3, R2+R4, R3+R4 and R2+R3+R4. R3 was the most frequent R gene, being present in all landraces. R4 was present in four and R2 in three of the landraces tested. Variation for resistance within landraces was limited except for landrace accessions ZP-0102, ZP-0109 and ZP-0137 which also showed variation for morphological traits. The resistance responses of landrace ZP-0109 were difficult to interpret, but suggested a genetic mixture with some evidence of less well documented R genes, R5 and/or R6, and possibly some unknown resistance to race 6.

INTRODUCTION

Pea bacterial blight, caused by *Pseudomonas syringae* pv. *pisi*, is a seedborne disease first recorded in the USA in 1915 (Sackett, 1916). Since then it has been reported in most pea growing areas of the world. Although the disease is thought to have been present in Spain for a considerable time (A. Ramos-Monreal, personal communication), there appears to be no published record of its occurrence.

Worldwide, seven races of *Ps. syr.* pv. *pisi* are currently recognized (Taylor *et al.*, 1989). The interaction of races and cultivars is controlled by a gene-for-gene relationship with avirulence genes in the pathogen matched by resistance genes in the host (Bevan *et al.*, 1995). Race specific resistance is widespread in commercial pea cultivars, however, there are no cultivars known to be resistant to race 6.

In Spain 70,000 ha. of dry peas were grown in 1994, including 21,000 ha. in Castilla y León (Northwest of Spain). The economic impact of *Ps. syr.* pv. *pisi* is most significant in this region of Spain, which was traditionally the main pea growing area. The disease is particularly prevalent in winter-sown crops which are exposed to more severe weather conditions and epidemics occur every four or five years resulting in

major crop losses.

Although 'modern cultivars' are now widely grown in Spain, traditional local varieties (landraces) also occur and collections of these are maintained by 'Centro de Recursos Fitogenéticos' (CRF), 'Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria' (INIA), Ministry of Agriculture, Madrid, Spain. Landraces have been described as primitive 'folk' varieties of traditional prescientific agriculture, antecedents of all modern varieties (Marshall, 1990). Genetic variability for disease resistance in landraces is thought to have developed in response to the continued coevolution of the crop and its parasites with the conscious or unconscious involvement of man. It was expected therefore that pea landraces would be genetic mixtures showing variability for a number of characters including disease resistance.

The objectives of the present study were to: (1) report the presence of *Ps. syr.* pv. *pisi* in Spain (2) determine the occurrence and frequency of race specific resistance to *Ps. syr.* pv. *pisi* in selected Spanish landraces, (3) compare resistance gene frequency in Spanish landraces with the known frequency in worldwide *Pisum* germplasm, (4) analyze genetic diversity both within and between landraces for resistance to *Ps. syr.* pv. *pisi* and morphological traits, and (5) identify potential new sources of resistance.

MATERIALS AND METHODS

Landrace accessions

A total of ten Spanish pea landraces were screened for resistance to *Ps. syr.* pv. *pisi*. Landraces were supplied by 'Servicio de Investigación Agraria' (SIA), 'Junta de Castilla y León', Valladolid, Spain, and were part of the Germplasm collection from CRF, Madrid. They were collected by INIA in 1978 and 1979 in different geographical areas of Spain with distinct climate and soil conditions and were considered to be representative of the traditional pea crop in Spain (Table 1 and Figure 1).

Two commercial cultivars, Kelvedon Wonder and Frisson, with known resistance characteristics, were included as controls in the comparison of variability within Spanish pea landraces.

Table 1. Accession number and geographical origin of the Spanish pea landraces (*Pisum sativum*)

Accession Number[a]	Accession Number[b]	Province	Town
ZP-0101	BG-999	Sevilla	La Roda
ZP-0102	BG-1004	La Coruña	Puentedeume
ZP-0103	BG-1010	León	Valle de Finolledo
ZP-0104	BG-1034	Castellón	Vall D'Alba
ZP-0106	BG-1056	Cádiz	Vejer de la Frontera
ZP-0107	BG-1077	Palencia	Boadilla de Rioseco
ZP-0109	BG-1100	Badajoz	Maguilla
ZP-0110	BG-1121	Murcia	Truyols
ZP-0112	BG-1153	Zamora	Santa Clara de Avedillo
ZP-0137	BG-2079	Oviedo	Quintana

[a] Number in the collection of 'Servicio de Investigación Agraria', 'Junta de Castilla y León', Valladolid, Spain

[b] Number in the germplasm bank of 'Centro de Recursos Fitogenéticos', INIA, Madrid, Spain

Fig. 1 Geographical origin of the Spanish landraces studied

Bacterial cultures

Seven isolates of *Ps. syr.* pv. *pisi* (Table 2) were used for resistance screening. They were the type strains corresponding to seven races of *Ps. syr.* pv. *pisi* (Bevan *et al.*, 1995). Further tests were made with two other isolates of race 6 (HRI-W 1683 and 1688).

In addition, 16 Spanish isolates were used for race typing. These isolates formed part of a collection made from a variety trial of winter peas in the Northwest of Spain in 1991. They were identified as *Ps. syr.* pv. *pisi* on the basis of pathogenicity to pea (cv. Kelvedon Wonder), reaction with specific antisera (Taylor, 1972; Lyons and Taylor, 1990) and race-typed by inoculation onto the standard differential cultivars (S.J. Roberts and J.D. Taylor, personal communication). They were race 6 (HRI-W 2815A, 2815B, 2815C, 4393, 4394, 4395, 4396, 4397) and race 4 (HRI-W 2817A, 2817B, 2817C, 4398, 4399, 4400, 4401, 4402).

Table 2. Source and origin of the type strains of *Pseudomonas syringae* pv. *pisi*

	Isolate Number		Origin		
Race	HRI-W	Source	Cultivar	Country	Year
1	299A	ICPM 2955	Rondo	New Zealand	1970
2	202	ICPM 815	Unknown	U.S.A.	1944
3	870A	HRI-W	Martus (seed)	U.S.A.	1975
4	895A	HRI-W	Martus (seed)	U.S.A.	1975
5	974B	HRI-W	Puget (seed)	U.S.A.	1978
6	1704B	MAFF	Stehgolt (seed)	France	1986
7	2491A*	ICPM 5316	Unknown	Australia	1976

HRI-W, Horticulture Research International, Wellesbourne, Warwickshire, U.K.
ICPM, International Collection of Microorganisms from Plants, Plant Diseases Division, DSRI, Auckland, New Zealand
MAFF, Ministry of Agriculture, Fisheries and Food, Cambridge, U.K.
*, Selection from ICPM 5316

Pathogenicity tests

Peas were sown in compost in seed trays and grown in a glasshouse at 18/16° C (day/night) with supplementary lighting to give 16 h day.

Pea seedlings were inoculated approximately two weeks after sowing, using a stem

inoculation procedure (Malik *et al.*, 1987). Isolates were cultured on King's Medium B (King *et al.*, 1954) for 24-48 h at 25°C. They were scraped from the surface of the plate with the tip of a sterile entomological mounting pin and stabbed into the main stem at its junction with the stipules at the youngest node.

The aim was to inoculate a minimum of 10 plants for each combination of accession and race. In practice, the number of inoculated plants varied between 13 and 30 per combination.

Plant reactions were recorded 7-10 days after inoculation and were assigned to one of three categories: a typical susceptible response showed an area of water-soaking spreading from the site of inoculation, whereas a typical resistant response resulted in necrosis localized at the point of inoculation. Incomplete expression of resistance was characterized by a combination of resistant and susceptible symptoms: localized necrosis surrounded by a limited water-soaked area.

Measurement of variation in morphological traits and resistance to pea blight races

An additional method of determining genetic diversity within and between landraces was to examine variation in morphological traits. The incidence of morphological variation was compared with genetic variation for resistance to *Ps. syr.* pv. *pisi*.

Data on five traits, selected as being independent of environmental influence and based on the evaluation of 10 plants per landrace and trait, were supplied by SIA, Valladolid, Spain. The traits considered were: flower colour, primary seed colour, shape of seed, degree of pod curvature and shape of pod tip.

Variation for inoculation responses to *Ps. syr.* pv. *pisi* within a landrace was calculated in two stages. First the reaction for each race was summarised as the percentage in the most common category (Resistance, Incomplete Resistance or Susceptbility). These percentages were then meaned across the races of the pathogen to give a description of uniformity. This was substracted from 100 to give a measure of variability.

RESULTS

Inoculation responses of the landraces to the seven races of *Ps. syr.* pv. *pisi* are shown in Table 3.

The validated gene-for-gene relationship between pea cultivars and races of *Ps. syr.* pv. *pisi*, (Bevan *et al.*, 1995) was used in combination with the resistance data obtained in the pathogenicity tests (response to the type strains) to infer the presence

of race specific resistance (R) genes (Table 4). R-genes were postulated on the basis of matching gene pairs, resistance gene(s) in the landrace and avirulence gene(s) in the pathogen race, which confers specific resistance to that race.

Table 3. Numbers of plants in Spanish pea landraces showing 'resistant: incomplete resistant: susceptible' responses (R:I:S) after inoculation with specific races of *Pseudomonas syringae* pv. *pisi*

Landrace	Race						
	1	2	3	4	5	6	7
	R:I:S	R:I:S	R:I:S	R:I:S	R:I:S	R:I:S	R:I:S
ZP-0101	30:0:0	0:0:30	18:0:0	26:0:0	14:0:0	0:0:18	19:0:0
ZP-0102	10:1:4	7:0:8	9:0:6	0:0:17	4:3:14	0:0:17	12:0:5
ZP-0103	19:0:0	0:0:18	15:4:2	0:0:14	0:0:15	0:1:17	16:0:0
ZP-0104	17:0:0	12:5:1	15:0:0	14:4:0	13:0:0	0:0:17	20:0:0
ZP-0106	17:0:0	0:0:13	15:3:0	0:0:16	0:0:16	0:0:14	15:1:0
ZP-0107	13:0:0	0:0:18	15:0:2	0:0:20	0:1:16	0:2:18	19:0:0
ZP-0109	9:2:5	0:0:21	8:0:13	0:0:17	4:5:9	7:8:5	3:0:17
ZP-0110	18:0:0	17:2:0	23:0:0	14:6:0	18:2:0	0:0:21	23:0::0
ZP-0112	13:0:0	0:0:18	14:0:0	17:2:0	11:7:0	0:1:20	17:0:1
ZP-0137	5:0:14	0:0:24	5:2:15	0:0:23	1:2:23	0:1:20	5:1:13
Frisson[a]	23:0:0	23:0:0	22:0:0	0:0:22	21:0:0	0:0:22	22:0:0
K. Wonder[b]	0:0:10	0:0:11	0:0:12	0:0:12	0:0:11	0:0:12	0:0:12

[a,b] commercial cultivars

The resistance phenotypes exhibited by the landraces mainly conformed to those previously described in pea cultivars (Taylor *et al.*, 1989; Bevan *et al.*, 1995) and corresponded to established genotypes R3, R2+R3, R3+R4, R2+R3+R4. The most common resistance gene was R3, being present in all 10 landraces. R4 was present in four and R2 in three of the landraces tested. None of the landraces was completely

susceptible or resistant to all races.

In general variation for resistance within landraces was not high and only three of them (ZP-0102, ZP-0109 and ZP-0137) showed major variation. The same accessions also showed variation in morphological traits (Table 5). Landrace ZP-0101 showed no variation either for resistance to *Ps. syr.* pv. *pisi* or any of the morphological traits studied. Landrace ZP-0109 showed the highest variability for resistance and was the only landrace which showed some resistance to race 6.

Table 4. Postulated resistance genes to *Pseudomonas syringae* pv. *pisi* in the Spanish pea landraces

Landrace	Race/avirulence (A) genes							Resistance (R) genes
	1	2	3	4	5	6	7	
	1	.	.	.	.	.	.	
	.	2	.	.	2	.	2	
	3	.	3	.	.	.	3	
	4	.	.	4	4	.	4	
	.	.	.	.	5*	.	.	
	6*	.	.	.	6*	.	.	
ZP-0101	-	+	-	-	-	+	-	R3+R4
ZP-0102	V	V	V	+	V	+	V	R2+R3
ZP-0103	-	+	-	+	+	+	-	R3
ZP-0104	-	-	-	-	-	+	-	R2+R3+R4
ZP-0106	-	+	-	+	+	+	-	R3
ZP-0107	-	+	-	+	+	+	-	R3
ZP-0109	V	+	V	+	V	V	V	R3+?
ZP-0110	-	-	-	-	-	+	-	R2+R3+R4
ZP-0112	-	+	-	-	-	+	-	R3+R4
ZP-0137	V	+	V	+	+	+	V	R3

+, predominantly susceptible; -, predominantly resistant/partially resistant; V, variable (some resistant and some susceptible) indicating genetic mixture; ., resistance gene absent; *, genes postulated but unproven (Bevan *et al.*, 1995)

For the five morphological traits recorded by SIA, Valladolid, all showed variability within landraces except the flower colour. This was clear evidence of genetic variation in addition to that shown for disease resistance. Eight landraces showed variation for

morphological traits, and although high variation in morphological traits could not be entirely correlated with high variation for resistance, landrace ZP-0109 appeared to be highly diverse for all the characters studied (Table 5).

Table 5. Comparison of variation for inoculation responses to *Pseudomonas syringae* pv. *pisi* (*Psp*) with morphological variation within Spanish pea landraces

Landrace/ Cultivar	Variation *Psp* (%)	Seed		Pod	
		Testa Colour	Shape	Degree of Curvature	Tip shape
ZP-0101	0.0				
ZP-0102	26.1			V	
ZP-0103	4.9			V	V
ZP-0104	7.9		V		
ZP-0106	3.3			V	V
ZP-0107	3.9	V		V	V
ZP-0109	29.5	V	V	V	V
ZP-0110	7.2		V		
ZP-0112	8.5				
ZP-0137	15.1		V	V	V
K.Wonder[a]	0.0				
Frisson[b]	0.0				

V, variable trait within a landrace, [a,b]commercial cultivars

Landrace ZP-0109

This was the only landrace to show resistance to race 6. In a first screening 15 out of 20 plants were resistant/partially resistant to race 6 (HRI-W 1704B). Resistant plants were reinoculated with two race 6 isolates (HRI-W 1683 and 1688). Although the degree of resistance varied slightly between isolates, resistance was confirmed in all cases.

To determine the type of resistance (race specific/race non-specific), the 15 resistant plants were further inoculated with race 2 (HRI-W 202) and all showed a susceptible reaction indicating that resistance to race 6, if genuine, was of a race specific nature. ZP-0109 was also the only landrace showing resistance to race 5 which was not controlled by either R2 or R4.

DISCUSSION

Although pea bacterial blight has been known in Spain for many years, there appears to be no published record of its occurrence. Isolations from Spain in 1991 were characterized as *Ps. syr.* pv. *pisi* and race-typed. Thus, this paper which is primarily concerned with resistance to a well established pathogen, also constitutes the first published record of the disease in Spain.

Landraces are expected to be genetic mixtures and show variability for certain characters. In the Spanish landraces tested, there was considerable variation for resistance to *Ps. syr.* pv. *pisi* between landraces. However, variation for resistance within landraces was less marked and only clearly present in three of them.

Amurrio *et al.* (1993) evaluated 72 Spanish pea landraces from the Northwest of the Iberian Peninsula. They found significant differences between landraces for all the characters considered except for seed hardness. They also observed variation within landraces but it was mainly present in qualitative traits. Results from the landraces tested in the present study suggested that they were genetic mixtures for several traits but only three of them showed evidence of variability for resistance to *Ps. syr.* pv. *pisi*. A homogeneous resistant response to a specific race of *Ps. syr.* pv. *pisi* within a landrace might suggest a selection pressure by that race of the pathogen.

The most common resistance gene found in the ten Spanish landraces was R3 which was present in all landraces studied. This is in agreement with the finding of R3 as the most common resistance gene in a worlwide *Pisum* sp. germplasm collection (Taylor *et al.*, 1989). The frequency of the R2 gene in Spanish landraces was also similar to that in the *Pisum* germplasm collection, however, the frequency of R3 and R4 was higher (Table 6). Furthermore, none of the Spanish landraces showed a complete absence of R-genes compared to 27% fully susceptible accessions in the *Pisum* germplasm collection. It has been shown (Hunter, 1996) that R2 is located on pea linkage group VII and that the balance of evidence indicates that R3 and R4, which are linked, are also located on linkage group VII. The frequency of the genotypes R2+3, R3+4 and R2+3+4 in Spanish and worldwide germplasm is thus in agreement with the known linkage data. Disease responses to race 5 in landrace ZP-0109 suggested that two less well documented genes, R5 and/or R6, could be operating in this instance or another unknown gene which also confers some degree of resistance to race 6.

Knowledge of the frequency of pea blight races in Spain is limited to the 16 isolates made in 1991, eight corresponding to race 6 and eight to race 4. Moreover, all of these isolates were obtained from a single region in Spain and may not be representative of the Spanish situation. In a more extensive study made in UK of the races found in pea seed stocks from 1987 to 1994 (Reeves *et al.*, 1996), race 2 was

most frequently recovered from infected seed stocks (65% to 92%), races 1, 3, 4, and 5 occurred infrequently in the samples examined. However, there was an increase in the incidence of recovery of race 6 over the period, representing 26% of the infected samples in 1994. In France, Schmit (1991) in tests on 50 isolates of *Ps. syr.* pv. *pisi* showed that the most common races were race 2 (52% of the isolates), race 6 (36%) and race 4 (12%). In this study Schmit differentiated between spring and winter cultivars: the most common race in spring cultivars was race 2, whereas in winter cultivars it was race 6 and to a lesser extent race 4. Resistance to race 2 is common in French winter cultivars but uncommon within the spring cultivars.

Table 6. Comparison of percentage frequency of Resistance (R) genes in 10 Spanish pea landraces with a representative collection of *Pisum sativum* germplasm

R-gene	Spanish Landraces	*Pisum sativum* Germplasm*
R0	0	27
R2	30	38
R3	100	56
R4	40	11

*Data calculated from 151 lines including cultivars, breeders lines and wild types (Taylor *et al.*, 1989)

By contrast in Australia in tests on 65 isolates (Hollaway and Bretag, 1995), the most common race was race 3 (64%) followed by race 6 (31%) and race 2 (5%). The importance of race 3 in Australia was probably due to the preponderance of a small number of race 3 susceptible cultivars. Indeed, 58% of the isolates studied came from a single race 3 susceptible source (cv. Dun or 'Dun type').

In the Northwest of Spain, the main area of the Spanish pea crop, spring cultivars are predominant. Nevertheless, the pattern of race distribution in an isolate collection from winter peas in Valladolid was the same as in winter-sown crops in the South of France where races 6 and 4 predominate. More isolations in different Spanish regions would need to be made to determine the incidence of the different races and make a comparison with the frequency of the resistance genes.

A common observation in studies in France and the UK was the predominance of the resistance gene R3 in *Pisum sativum* germplasm and the very low frequency of races carrying the avirulence gene A3 (races 1, 3 and 7) in disease outbreaks. However, the potential of race 3, in the absence of the R3 resistance gene, has been

clearly demonstrated in Australia (Hollaway and Bretag, 1995). A common feature in all studies (ie. in Europe and Australia) was the lack of resistance to race 6 in commercial cultivars and the apparent increase in importance of race 6. For the host/pathogen combination *Phaseolus vulgaris/Pseudomonas syringae* pv. *phaseolicola*, the resistance gene R4 which shows the highest R-gene frequency (35%) in *Phaseolus vulgaris* germplasm, is matched by the lowest corresponding avirulence gene frequency (3.5%) (Taylor *et al.*, 1996). Therefore, it could be suggested that the history of past epidemics is reflected in the frequency of race- specific resistance genes.

The increasing importance of race 6 may be explained by the apparent absence of resistance to this race in commercial cultivars. For this reason it is important to identify sources of resistance to race 6 for future breeding programmes. This might be obtained from the Spanish landrace ZP-0109, or from a source of race non-specific resistance identified in *Pisum abyssinicum* (Schmit *et al.*, 1993; Elvira-Recuenco and Taylor, 1998)

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Chapter 3

Inheritance of race non-specific resistance to *Pseudomonas syringae* pv. *pisi* derived from *Pisum abyssinicum* and molecular markers for resistance

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ABSTRACT

Inheritance of potential race non-specific resistance (RNSR) to pea bacterial blight present in *Pisum abyssinicum* was studied through a crossing programme between two *P. sativum* cultivars ('Kelvedon Wonder', without race specific resistance, and 'Fortune' with three race specific resistance genes) and two *P. abyssinicum* accessions (JI2202 and JI1640). F2 ratios in crosses with 'K. Wonder' generally indicated a single recessive inheritance of RNSR, while crosses with 'Fortune' produced an excess of resistant plants for this hypothesis. However, F3 plants produced from selected resistant and susceptible F2s did not segregate as expected for a single recessive gene, but in a complex pattern conforming to a series of ratios 13:3, 1:1, 3:13 (Resistant:Susceptible) or all susceptible. In an attempt to clarify the F3 segregation a molecular approach was taken using a *Ty1-copia* group retrotransposon of pea (PDR1) and SSAP (sequence specific amplification polymorphisms) markers. Three markers cosegregating with resistance and three cosegregating with susceptibility were identified. Two of the *P. sativum* markers were mapped using two recombinant inbred populations locating the susceptible allele to linkage group V in *P. sativum*. Although the apparent complexity of the segregation could potentially be affected by karyotypic divergence between *P. sativum* and *P. abyssinicum*, results in this study point to a model of inheritance which is not polygenic, but more likely a major recessive gene together with a number of modifiers. The exploitation of the *P. abyssinicum* resistance has been initiated through a crossing programme of selected resistant F5s to a range of commercial *P. sativum* cultivars.

INTRODUCTION

Pea bacterial blight (*Pseudomonas syringae* pv. *pisi*) is a seed borne disease that was first recorded in 1915 (Sackett, 1916) and occurs worldwide. Serious losses are caused by seedling infection, particularly in winter-sown crops, under cool and wet conditions, when lesions girdling the stem may lead to complete plant collapse.

Seven races are currently recognized (Taylor *et al.*, 1989) and the interaction of races and cultivars is controlled by a gene-for-gene relationship that confers race specific resistance (Bevan *et al.*, 1995). There are no cultivars known to be resistant to race 6. Races 2, 4 and 6 are reported to be the predominant races in Europe, however, race 6 has become increasingly important (Schmit, 1991; Reeves *et al.*, 1996), possibly due to the employment of cultivars resistant to races 2 and 4.

Screening for stem resistance to pea bacterial blight in *Pisum* germplasm as part of a CEC programme, showed that all accessions listed as *Pisum abyssinicum* were resistant to all races of *Ps. syr.* pv. *pisi* including race 6 (Schmit *et al.*, 1993; Elvira-Recuenco and Taylor, 1998). This suggested a potential race non-specific resistance

(RNSR). In total sixteen accessions of *P. abyssinicum* originating from Ethiopia and one from Yemen were identified, all of which were resistant to all races. Ethiopia is one of the primary centres of diversity of cultivated plants, characterized by the isolation of agriculture among the mountains, the fairly ancient type of agriculture and the vicinity to the main centres of origin of 'old world'crops including peas (Vavilov, 1992).

Pisum abyssinicum is found mainly in northern Ethiopia in the high mountains forming the eastern escarpment in Tigre and Wollo provinces (Westphal, 1972). Govorov (1930) found it mixed with other peas, chickpea (*Cicer arietinum*) and horse bean (*Vicia faba*) in the regions of Adis Abeba and Hararge. He also pointed at introgression between the two groups of Ethiopian peas (*P. sativum* and *P. abyssininicum*). Gentry (1971) mentioned the occurrence of a seed type which suggested hybridization between both types.

Due to the absence of specific resistance to race 6 and the possibility of the appearance of new races, the RNSR present in *P. abyssinicum* offers the possibility to achieve a potentially durable resistance to all races of *Ps. syr.* pv. *pisi*. In *Phaseolus vulgaris* a similar type of RNSR to all races of *Ps. syr.* pv. *phaseolicola* has been identified. In this system it has been suggested that the combination of RNSR with one or more dominant race specific genes may provide an additive and durable resistance (Taylor *et al.*, 1996).

When this study was initiated the inheritance of RNSR to pea blight derived from *P. abyssinicum* was largely unknown, although evidence from some preliminary crosses suggested a recessive nature of its inheritance and indicated that resistance could be transferred from *P. abyssinicum* to *P. sativum*. If this resistance could be stabilized in a *P. sativum* background and the mode of inheritance determined then it could be successfully used in breeding programmes.

The main objective of the work presented in this paper was to study the mode of inheritance of RNSR to pea blight derived from *P. abyssinicum* in a *P. sativum* background. In an attempt to clarify the complex segregation patterns that became apparent at F3, a molecular approach was initiated using a marker system based on a *Ty1-copia* group retrotransposon of pea (PDR1) and the SSAP (sequence-specific amplification polymorphism) technique. This was known to be particularly informative for both diversity studies and linkage analysis in pea (Ellis *et al.*, 1998). An additional benefit was the identification of molecular markers for resistance of possible use in future breeding programmes.

MATERIAL AND METHODS

Plant material

The *Pisum* accessions used in this study are shown in Table 1. Specific crosses made to elucidate the inheritance of potential RNSR were made between two *Pisum sativum* cultivars, Kelvedon Wonder (KW) and Fortune (FORT), and two *Pisum abyssinicum* accessions, JI2202 and JI1640. 'K. Wonder' is susceptible to all races of *P. s.* pv. *pisi*, 'Fortune' is resistant to all races except race 6, JI2202 and JI1640 are partially resistant in the stem to all races (potential RNSR).

Plants for crossing, resistance scoring and seed production from selected plants after scoring, were grown in a glasshouse at 20 ± 2° C day/18 ± 2° C night with supplementary light up to 12h. For bulk multiplication, seedlings were grown in a glasshouse and after 2 weeks transplanted to field soil under polythene tunnel structures. Because some of these plants were tall or climbing types, they were supported by canes or netting.

Pathogenicity tests

For resistance screening to race 6, pea seedlings were inoculated with isolate 1704 B (HRI collection, Wellesbourne, UK) approximately two weeks after sowing, using a stem inoculation procedure (Malik *et al.*, 1987). Isolates were cultured on plates of King's medium B (King *et al.*, 1954) for 24-48 h at 25° C. They were scraped from the surface of the plate with the tip of a sterile entomological mounting pin and stabbed into the main stem at its junction with the stipules at the youngest node (not expanded). Parental types were used in all tests as positive and negative controls: K. Wonder and Fortune for susceptible responses; JI2202 and JI1640 for resistant responses.

Plant reactions were recorded 7-10 days after inoculation. A typical susceptible response showed an area of water-soaking spreading from the site of inoculation, whereas a typical resistant response resulted in necrosis localized at the point of inoculation. Inoculation responses were assigned to one of three categories: (1). Susceptible, with water-soaking or weak water-soaking but no necrosis; (2). Intermediate, with weak water-soaking and necrosis, (this category may have comprised heterozygous plants where the expression of resistance was influenced by environmental conditions); and (3). Resistant, with necrosis or weak necrosis but no water-soaking.

Table 1. Resistance characteristics to *Pseudomonas syringae* pv. *pisi* of the pea accessions used in this study

Accession number[a]	Stem resistance to races:	Inferred Resistance Gene(s)[b]
Pisum sativum		
JI15 (WBH 1458)	1	R1 (*Ppi*-1)
JI281 (ex Ethiopia)	2,5,7	R2 (*Ppi*-2)
JI399 (cv. Cennia)	None	None
JI2430(cv. Kelvedon Wonder)	None	None
JI2439 (cv. Fortune)	1,2,3,4,5,7	R2, R3, R4 (*Ppi*-2, *Ppi*-3, *Ppi*-4)
Pisum abyssinicum		
JI2 (ex Ethiopia)	1,2,3,4,5,6,7	Race non-specific resistance
JI130 (ex Ethiopia)	1,2,3,4,5,6,7	Race non-specific resistance
JI225 (ex Ethiopia)	1,2,3,4,5,6,7	Race non-specific resistance
JI1556 (ex Ethiopia)	1,2,3,4,5,6,7	Race non-specific resistance
JI1640 (ex Ethiopia)	1,2,3,4,5,6,7	Race non-specific resistance
JI2202 (ex Yemen)	1,2,3,4,5,6,7	Race non-specific resistance
JI2385 (ex Yemen)	1,5	(R6)

[a] Accession number in the *Pisum* germplasm collection, John Innes Centre, Norwich, UK

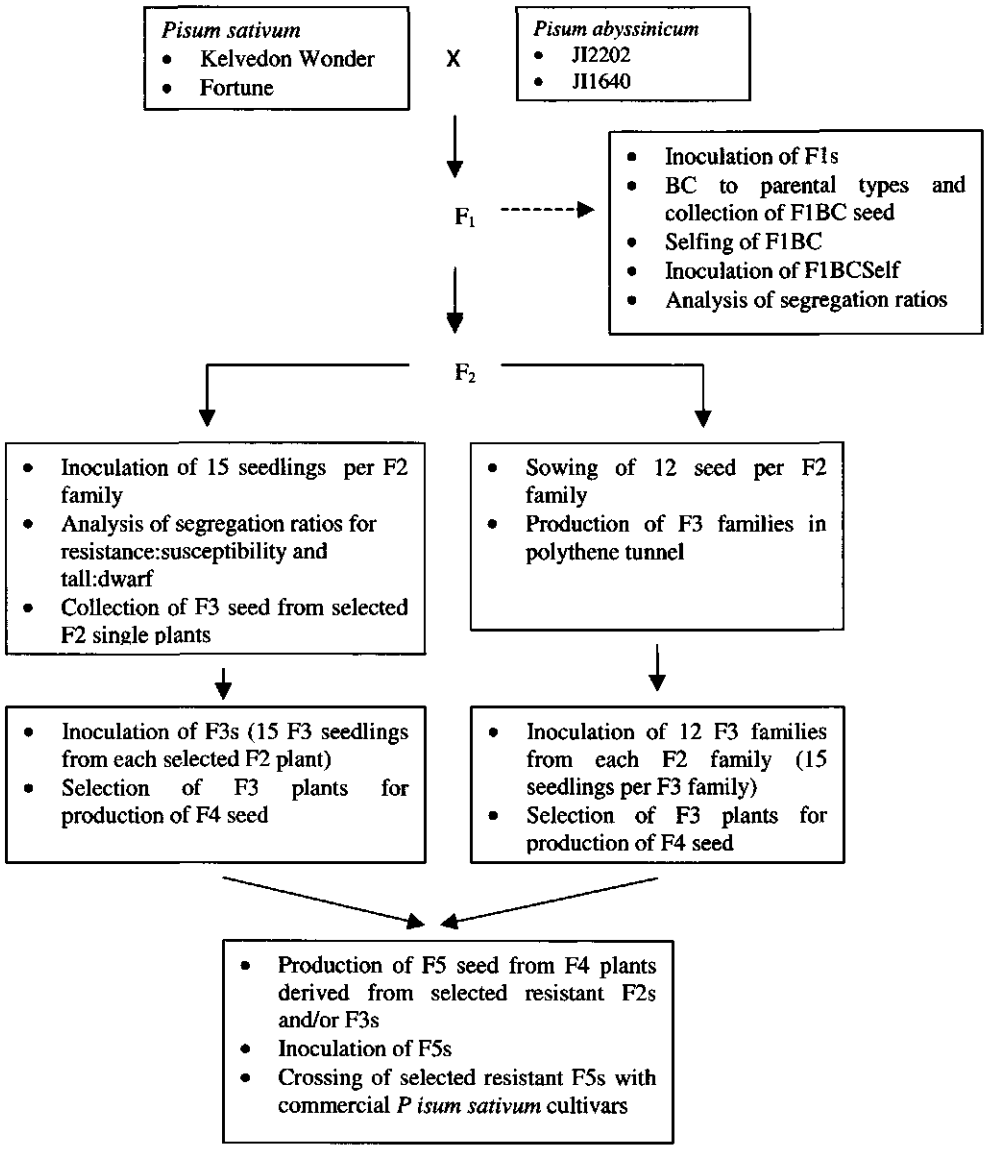
[b] Designation of race specific resistance genes (Bevan *et al.*, 1995)

Inheritance studies

A total of 275 reciprocal crosses were made between accessions of *P. sativum* and *P. abyssinicum* according to the general scheme outlined in Fig. 1. F1 seed was harvested and selfed seed discarded. F1 seeds were sown to produce F2s, which were collected separately from each F1 plant.

A random selection of the F2s (66 families) was tested for resistance. For each family approximately 15 seedlings were stem inoculated with race 6 as described above. Morphological characters including plant height, flower colour and seed pigmentation were also recorded. The numbers of plants in each resistance response category were recorded, the segregation ratios determined and the probability calculated for various hypotheses. Probability was also calculated for the hypothesis 3:1 (Tall:Dwarf). A selection of resistant, partially resistant and a limited number of susceptible F2 seedlings were grown on for the production of F3 seed. F3 seeds were sown (15 seed per F3 family) in a glasshouse and the seedlings inoculated with race 6.

Fig 1. Crossing programme and resistance screening to race 6



In addition, seeds of 23 F2 families (12 seeds per family) were grown in field soil under a polythene tunnel structure to produce F3 seed. For 18 of these F2 families the segregation for stem resistance to race 6 was already known from previous tests on the same F2 families. F3s produced in tunnels derived from 9 F2 families (12 F3 families per F2 family, 15 seeds per F3 family) were sown in a glasshouse and seedlings inoculated with race 6. Reactions of F3 seedlings were scored and segregation ratios analyzed. Plant height was also recorded. F3 seedlings were selected, mainly those with a resistant response, transplanted and F4 seed harvested from *ca* 400 plants.

Further selection of F4s was made on the basis of the inoculation responses of the previous generations (F2s and F3s). F4s derived from F2 and F3 families showing the highest proportion of resistant plants were taken forward for F5 seed production. For this, 35 F4 families (3 seeds per family) were grown under a polythene tunnel structure and F5 seed was collected from individual plants. Thirty of these F5 families (8-10 seedlings per family) were stem inoculated with race 6. Plants showing a strong resistant response were selected and crossed to the parental lines of the present study, as well as to the commercial cultivars Solara (with race specific resistance gene R3), Belinda (R3), Cea (R3), Esla (R3), Frisson (R2 and R3), Fortune (R2, R3 and R4), Ballet and Orb (unknown resistance) and to the accession JI15 (R1).

The first generation (11 F1s, 2-3 seeds per F1) from crosses between K. Wonder and *P. abyssinicum* accessions (JI2202 and JI1640) made prior to the present study, were stem inoculated with race 6 and used for backcrossing with 'K. Wonder'. F1BC plants were multiplied under a polythene tunnel structure and scored for growth habit, leaf dentation, flower colour, pod length and seed characteristics: pigmentation, size, wrinkled/round and hilum colour. Selfs were discarded, seed was harvested from F1BC plants and 20 seed from each F1BCSelf were sown and screened for resistance to race 6.

Molecular markers for resistance and genetic mapping

Screening of parental lines with the PDR1-SSAP method

Amplification products corresponding to insertion sites of the pea *Ty1-copia* class retrotransposon PDR1 were obtained for the *P. sativum* parental lines, K. Wonder and Fortune, using the PPT (polypurine tract) primer and Taq adapter primer with TT as selective bases (as described in Ellis *et al.*, 1998). These were compared with the products obtained for the *P. sativum* accession JI399, to which they were very similar. Map positions for 60 such markers in the JI15 x JI399 recombinant inbred population and 61 markers in the JI281 x JI399 RI population using the PDR1-SSAP method

have been described by Ellis *et al.* (1998).

The *P. abyssinicum* parental lines, JI2202 and JI1640 (10 plants per line), were compared with the *P. abyssinicum* accessions JI2, JI130, JI225, JI1556 and JI2385, which had been screened by Ellis *et al.* (1998), who found a very low diversity within the *P. abyssinicum* group for these PDR1 markers. The *P. abyssinicum* markers were in turn compared to those derived from the *P. sativum* lines.

Screening of the offspring with the PDR1-SSAP method

Selection of plant material for DNA extraction

Segregant F3, F4 and F5 populations of the crossing programme described above were used for molecular marker analysis. It was aimed to screen single plants that were homozygous for resistance or susceptibility. The plant material used for the selection of these plants was:

1. Potentially homozygous resistant plants

1.a. From the F4 families of the crosses KW x JI2202 (65 families) and FORT x JI2202 (39 families), two seeds were grown per family, these had a known resistance background in the corresponding F3s, and for some this was also known for the F2s.

1.b. Five F5 plants resistant to race 6, derived from resistant F2 and F3 families.

2. Potentially homozygous susceptible plants

2.a. F3 families (2 seed per family) of the crosses KW x JI2202 (6 families) and FORT x JI2202 (3 families). These families were previously screened by inoculation of 15 seed per family and all plants within a family showed to be susceptible.

2.b. F3 individuals that were progeny tested for homozygosity: 5 families of the cross KW x JI2202 and 4 families of the cross FORT x JI2202, 16 seeds were grown per family.

A total of 275 seedlings were inoculated with race 6. Selection of lines for further molecular analysis was according to these inoculation responses and resistance background in previous generations. Material selected for DNA extraction is shown in Table 2. Additional DNA extractions were made from the parental lines 'K. Wonder', 'Fortune', JI2202 and JI1640, and the accessions JI399, JI2, JI2385.

DNA extraction

Leaflets were collected from 14 day old seedlings and stored at –20° C. One or two leaflets (*ca.* 0.1 g fresh weight) were placed in 1.5 ml eppendorfs, submerged in liquid

nitrogen and ground using an eppendorf mixer. DNA was extracted with the Nucleon Phytopure Kit and protocol (Amersham, Life Science). For the parental lines, this was done using 0.4-0.5 g fresh tissue from a single plant. DNA concentration and purity was estimated spectrophotometrically and from ethidium bromide fluorescence of DNA after electrophoresis in 0.8% agarose gels.

Table 2. Number of plants selected for DNA extraction from each cross and generation

Cross/generation	resistant	partial resistant	susceptible
KW x JI2202			
F3			5
F4	10	9	
F5	5		
FORT x JI2202			
F3			3
F4	6	9	

The PDR1-SSAP method

The protocol used in this study is based on the protocol described by Ellis *et al.* (1998). Modifications of the method for each step were as follows:

*Taq*I digestion

Genomic DNA (0.5 µg) was digested with 25 U *Taq*I (Gibco) in a 50 µl mixture containing BSA (0.1mg/ml) and *Taq*I buffer (50 mM Tris-HCl pH 8.0, 10mM $MgCl_2$, 50 mM NaCl) and incubated at 65°C overnight.

Ligation of Taq adapter

A 40 µl solution containing 50 pmol of Taq adapter, 4 mM ATP and 1 U of T4 DNA Ligase in RL buffer (10 mM Tris-acetate pH 7.5, 10 mM magnesium acetate, 50 mM potasium acetate, 5 mM DTT) was added to 10 µl of the *Taq*I digested DNA and incubated at 37° C overnight. The 50 µl digested/ligated DNA was diluted adding 100 µl of 0.1 x TE (10 mM Tris-Cl, 0.1 mM EDTA pH 8) and stored at 4° C.

PCR conditions and gel analysis of PCR products

These were as previously described (Ellis *et al.*, 1998) and the selective bases used wit the Taq primer were TT, TA, TC, AT and AA.

RESULTS

Inheritance studies

Crossing success rate and F1 fertility

A total of 275 crosses were made and 104 of these were successful (Table 3). The percentage of successful crosses varied from 15 % to 57 % depending on the parents. Crossing success rate was generally higher for crosses involving 'Fortune' than for crosses involving 'K. Wonder'. F1 fertility (referred to successful F2 seed in Table 3) was lower for crosses involving *P. abyssinicum* as the female parent, particularly for JI1640, this also presented lower crossing success rates than JI2202 when used as female parent.

Table 3. Total number of crosses, crossing success and F1 fertility

Cross Female x Male	No. of Crosses	Successful Crosses (%)	Successful F2 seed (%)
KW x JI2202	60	19 (32)	17 (89)
KW x JI1640	21	7 (33)	7 (100)
FORT x JI2202	47	23 (49)	22 (96)
FORT x JI1640	21	12 (57)	12 (100)
JI2202 x KW	42	18 (43)	12 (67)
JI2202 x FORT	39	16 (41)	8 (50)
JI1640 x KW	26	4 (15)	2 (50)
JI1640 x FORT	19	5 (26)	0 (0)

F2 segregation

The ratios of Resistant:Susceptible (R:S) plants in the majority of F2 families

suggested a monogenic recessive inheritance of resistance. Segregation ratios (Table 4) were analyzed for the null hypothesis 1:3 (R:S) considering two possibilities for the calculation of probabilities: the intermediate response category was considered to be either (1) resistant or (2) susceptible. One F2 family was omitted since all plants within the family were susceptible.

Table 4. Segregation for resistance to *Ps. syr.* pv. *pisi* race 6 in F2s of crosses between the *P. sativum* cultivars Kelvedon Wonder and Fortune and the *P. abyssinicum* accessions JI2202 and JI1640 and probabilities for the hypothesis 1:3 Resistant (R):Susceptible (S) when the intermediate response category is included either as resistant or susceptible

	KW x JI2202	JI2202 x KW	KW x JI1640	JI1640 x KW	FORT x JI2202	JI2202 x FORT	FORT x JI1640
No.F2 families	11	11	7	5	13	8	10
Susc.[a]	101	60	54	23	90	45	75
Res.[b]	38	43	21	7	52	22	38
χ^2 1:3	0.41	15.41	0.36	0.08	10.23	2.19	4.49
P	0.5-0.8	<0.001	0.5-0.8	0.90-0.95	0.001-0.01	0.1-0.2	0.02-0.05
Susc.[c]	114	78	63	26	108	58	101
Res.[d]	25	25	12	4	34	9	12
χ^2 1:3	3.65	0.03	3.24	2.18	0.08	4.78	12.46
P	0.05-0.1	0.8-0.9	0.05-0.1	0.1-0.2	0.90-0.95	0.02-0.05	<0.01
Tall[e]	103	87	55		99	55	81
Dwarf[f]	34	17	22		43	12	32
χ^2 3:1	0.002	4.15	0.52		2.11	1.80	0.66
P	0.95-0.98	0.02-0.05	0.2-0.5		0.1-0.2	0.1-0.2	0.2-0.5

[a,b] Number of susceptible/resistant plants when intermediate plants are included in the resistant category; [c,d] Number of susceptible/resistant plants when intermediate plants are included in the susceptible category; [e,f] Number of tall/dwarf plants. As a measure of the normality of the cross, observations were also made of a morphological marker (height)

In the crosses with 'K. Wonder', with the exception of JI2202 x KW, the ratio R:S was 1:3 (P>0.05), with higher *P* values (P>0.5) when the intermediate response

category was considered as resistant. In the same crosses, the segregation ratio for the morphological marker plant height was 3:1 Tall:Dwarf (P>0.2). In JI2202 x KW, the R:S ratio was 1:3 (P>0.8) only when the intermediate category was considered as susceptible. In the same cross the plant height character did not fit the expected ratio 3:1 T:D (P<0.05). Also this cross generated more F1 plants failing to set F2 seed than its reciprocal which could have contributed to the odd segregation ratios.

Crosses with 'Fortune' behaved somewhat differently. There was a higher number of resistant plants than in crosses with 'K. Wonder'. In one cross where the intermediate plants were incorporated in the resistant category there was an acceptable fit to a 1:3 R:S ratio. In another cross it required the intermediate plants to be incorporated into the susceptible category to give a fit to 1:3. In a third cross incorporating the intermediate plants into either the resistant or susceptible category failed to give a fit to a 1:3. This is clearly unsatisfactory but it may reflect the influence of other resistance genes (race specific) from 'Fortune' (R2, R3 and R4) on the expression of resistance.

The number of plants that were dwarf and resistant was higher than would be expected for independent assortment. In the crosses KW x JI2202, KW x JI1640 and JI2202 x FORT where $P>0.05$ for the hypothesis 1:3 (R:S) and 3:1 (T:D), the probability was calculated for the null hypothesis 9:3:3:1, (TS:TR:DS:DR). Only for the cross JI2202 x FORT did the probability value indicate a potential linkage (Table 5).

Table 5. Observed and expected numbers for the phenotypes tall-susceptible (TS), tall-resistant (TR), dwarf-susceptible (DS) and dwarf resistant (DR) and probabilities for independent assortment 9:3:3:1 in F2s

Cross[a]/Phenotypes...		TS	TR	DS	DR
KW x JI2202	Obs.	78	23	19	14
	Exp.	75.4	25.1	25.1	8.4
	χ^2, *P*	5.48, 0.1-0.2			
KW x JI1640	Obs.	37	10	11	7
	Exp.	36.6	12.2	12.2	4
	χ^2, *P*	2.77, 0.2-0.5			
JI2202 x FORT	Obs.	43	12	2	10
	Exp.	37.7	12.6	12.6	4.1
	χ^2, *P*	18.18, <0.001			

[a] Only the crosses where the ratios 1:3 R:S and 3:1 T:D gave $P>0.05$ were included in this analysis

F3 segregation

F3s produced from selected F2s

The observed pattern of segregation from F2 data (Table 4) suggested that resistance derived from *P. abyssinicum* was controlled in a recessive manner, most probably a single recessive gene. On this basis, it was expected that F3 families derived from resistant F2 plants would be homozygous resistant. However, the observed segregation pattern showed three segregating categories for F3s derived from resistant F2s and two segregating categories for F3s derived from susceptible F2s. The segregating categories (R:S) had a tendency to 13:3 or 3:1, 9:7 or 1:1 and 3:13 or 1:3. Chi-square tests for these hypotheses showed that the most likely segregation ratios were either 13:3 or 1:1 or 3:13 R:S. For the F3s produced from susceptible F2s, they were all susceptible or segregating either 1:1 or 3:13 R:S (Tables 6 and 8).

The ratios of the different F3 categories [all R]:[13:3]:[1:1]:[3:13]:[all S] were 0:5:11:9:0 for F3s derived from resistant F2s and 0:0:2:9:3 for F3s derived from susceptible F2s. Thus, category 13:3 R:S is only present in F3s derived from resistant F2s, number of families falling in the 1:1 category is also higher for F3s derived from resistant F2s and the category with all plants susceptible is only present in F3s derived from susceptible F2s. There were no F3 families in which all plants were resistant. All the F3 families in the 13:3 R:S category were either dwarf or segregating for height but never tall.

F3s produced from unselected F2s

For the F3's derived from F2 plants that were not inoculated with race 6, three different F3 segregating categories were observed fitting the ratios either 13:3 or 1:1 or 3:13 R:S as for the F3s derived from selected F2s with a known response to race 6 (Tables 7 and 8). Approximately half of the F3 families were uniformly susceptible (all plants susceptible within a family), however, only 2 families were uniformly resistant (in crosses with 'Fortune'). The ratios of the different F3 categories [all R]:[13:3]:[1:1]:[3:13]:[all S] were 2:2:17:25:43. The resistant and 13:3 families were all dwarf. From both sets of F3 data the three observed segregation ratios were present in progenies derived from the same F1 plants. There was therefore no possibility that variation in the genetic constitution of individual parental plants used in the crossing programme contributed to the different ratios observed at F3.

seedlings died. If resistance is controlled by a single recessive gene, half of the F1BCSelf families were expected to segregate for resistance and half to be uniformly susceptible. For plant height half of the families were expected to segregate for height and half to be uniformly dwarf. The results obtained showed the expected segregation for height, however, all the six F1BCSelf families were uniformly susceptible and none of them segregated for resistance (Table 10). This suggests that there might be other gene(s) modifying the resistance present in *P. abyssinicum*.

Table 8. Observed Resistant:Susceptible ratios and probabilities for each of the three F3 segregating categories when F3 families were either derived from selected F2s or unselected F2s

	F3s from selected F2s		F3s from unselected F2s
	From res. F2	From susc. F2	Not tested at F2
Obs. R:S	61:10		26:3
13:3 χ^2, *P*	1.0, 0.2-0.5		1.31, 0.2-0.5
3:1 χ^2, *P*	1.6, 0.1-0.2		3.29, 0.05-0.1
Obs. R:S	70:77	14:13	111:111
9:7 χ^2, *P*	4.4, <0.05	0.21, 0.5-0.8	3.49, 0.05-0.1
1:1 χ^2, *P*	0.34, 0.5-0.8	0.036, 0.8-0.9	0, 0.99
Obs. R:S	19:99	17:96	58:294
3:13 χ^2, *P*	0.42, 0.5-0.8	0.99, 0.2-0.5	1.19, 0.2-0.5
1:3 χ^2, *P*	4.98, <0.05	5.97, <0.02	13.6, <0.001

Table 9. Number of resistant and susceptible plants to *Ps. syr.* pv. *pisi* race 6 in F4s derived from resistant selections from the F3 segregating categories 13:3, 1:1 and 3:13 R:S

F3 category	Resistant:Susceptible at F4	
	KW x JI2202	FORT x JI2202
13:3	45:6[a]	59:7
1:1	28:10	55:23
3:13	4:15	8:9

[a] Numbers of resistant or susceptible plants were obtained from a series of F4 families (2-4 plants per family) screened from each segregating category and cross.

Table 10. Number of resistant and susceptible plants (R:S) to *Ps. syr.* pv. *pisi* race 6 and tall/dwarf (T:D) plants for each F1BCSelf family

Cross	Backcross	F1BCSelf	
female x male	female x male	R:S	T:D
KW x JI2202	KW x F1	0:20	0:20
	KW x F1	0:20	12:8
KW x JI1640	KW x F1	0:20	0:20
	KW x F1	0:19	14:6
	KW x F1	0:20	14:6
	KW x F1	0:20	0:20

Summary of segregation results

The F1 was generally uniformly susceptible as expected. There were reciprocal differences in F1 plant fecundity and segregation patterns at F2, which were predominantly 1:3 R:S. However, segregation patterns at F3 did not conform to the expected segregations for a single recessive gene, since segregation patterns appeared to fall into three different ratios (13:3, 1:1, 3:13). The apparent stabilization of resistance in true breeding populations at F5 together with the fairly common 1:3 segregation at F2 suggest that at most only a small number of genes is likely to be responsible for the resistance trait. In order to provide a simplifying framework within which these data could be understood, a molecular marker approach was taken to investigate these segregating patterns.

Molecular markers for resistance and genetic mapping

Screening of parental lines

An estimation on the number of markers (polymorphic bands) was initially made with the selective bases TT for a pairwise comparison between the parental lines ('K. Wonder', 'Fortune' and JI2202) and JI399 (Table 11). There was a relatively low polymorphism between the *P. sativum* lines and no polymorphism within the *P. abyssinicum* lines except for one of the JI1640 plants, which lacked one of the bands. However, polymorphism between *P. sativum* and *P. abyssinicum* was very high (Fig. 2).

These results show that the PDR1 product banding patterns were very different

between *P. sativum* and *P. abyssinicum*, and therefore many markers will segregate in the offspring of crosses between them. The fact that JI399 is not very different from 'K. Wonder' and 'Fortune' suggests that a high proportion of polymorphic bands cosegregating with susceptibility in these crosses will correspond to JI399 bands. Hence as JI399 is a parent of two RI mapping populations, there is a reasonable likelihood that their genetic map position will be known.

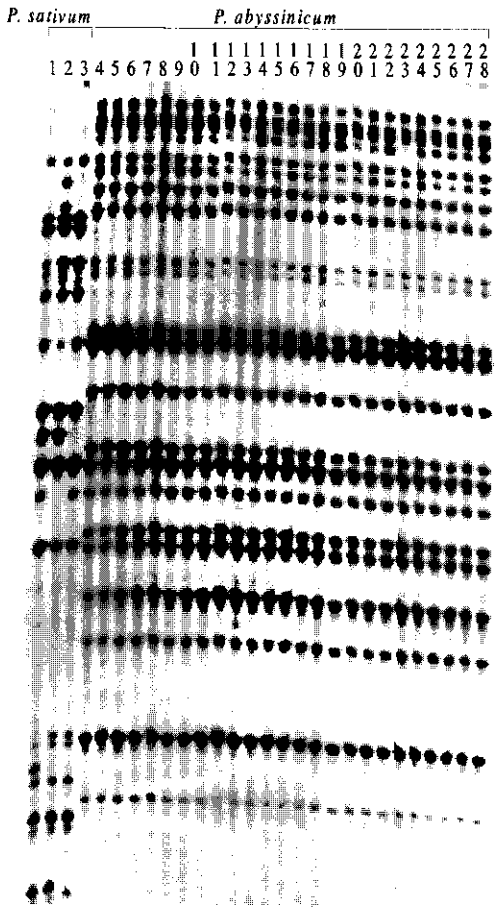


Fig. 2. PDR1 PPT primed SSAP products, when using the selective bases TT with the Taq adapter, from the parental lines of the present crossing programme and other *Pisum* lines. Lane 1: JI399 (parental line of two mapped recombinant inbred populations); lane 2: Kelvedon Wonder, lane 3: Fortune (*Pisum sativum* parenal lines); lanes 4 –13: JI2202, 14-23: JI1640 (*P. abyssinicum* parental lines); lane 24: JI2; lane 25: JI130; lane 26: JI225; lane 27: JI1556 and lane 28: JI2385

Table 11. Number of band differences in pairwise comparisons of the parental lines from the crossing programme (cvs. K. Wonder, Fortune and *P. abyssinicum* accession JI2202) and the parental line (JI 399) of two RI populations with the selective bases TA, TC, TT and AA (PDR1-PPT method)

	KW-JI2202	FORT-JI2202	JI399-JI2202	KW-FORT	KW-JI399	FORT-JI399
TA	13	13	15	4	4	6
TC	19	19	20	0	1	1
TT	31	29	29	6	5	5
AA	27	22	23	8	4	5
Sum	90	83	87	18	14	17

Screening of the offspring

Sampling of the genome with the PDR1-SSAP method

Estimation of total number of markers (polymorphic bands) with the selective bases tested gives an indication of how extensive is the sampling of the genome using the PDR1 markers (Table 11). These markers have been shown to be well distributed throughout the pea genome (Ellis *et al.*, 1998).

Markers for resistance

Three *P. abyssinicum* bands that cosegregated with resistance were identified with the bases TA, AT and AA and three *P. sativum* bands that cosegregated with susceptibility were identified with the selective bases AA and TC (Table 12 and Fig. 3).

All of the plants tested in which the three *P. abyssinicum* bands were present and the three *P. sativum* bands were absent, were resistant. These plants mainly derived from resistant selections from the 13:3 R:S F3 category. By comparison half of the resistant plants in which some of the *P. sativum* bands were present, were derived from 1:1 and 3:13 R:S F3 categories.

Table 12. Number of susceptible (S) and resistant (R) plants with a polymorphic band (present/absent) at F3/F4/F5 of the crosses between K. Wonder or Fortune with JI2202 for the selective bases TA, AT, AA and TC (PDR1-SSAP method)

	Band origin	Present S	Present R	Absent S	Absent R
TA(a)	*abyssinicum*	1	29	9	5
AT(b)	*abyssinicum*	1	29	9	5
AA(c)	*sativum*	6	6	4	26
AA(d)	*abyssinicum*	1	26	9	6
TC(e)	*sativum*	7	7	3	27
TC(f)	*sativum*	9	10	1	24

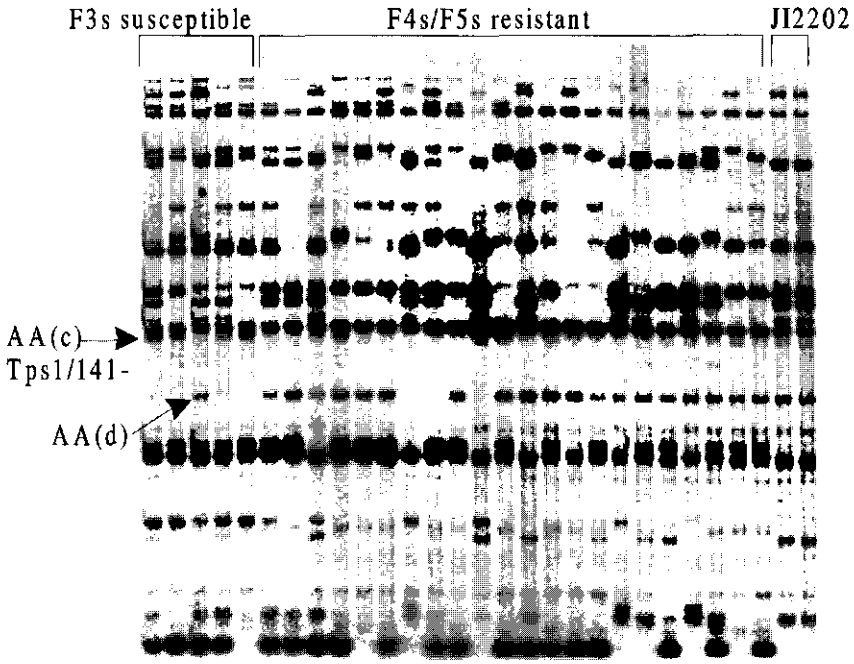


Fig. 3. PDR1 PPT primed SSAP products, when using the selective bases AA with the Taq adapter, from the segregating populations of the cross Kelvedon Wonder x JI2202. Two markers have been identified: AA(c), cosegregating with susceptibility mapped to Tps1/141- and AA(d) cosegregating with resistance

Estimation of linkage

Number of parental individuals for a *P. sativum* marker was calculated as the sum of those individuals that were susceptible and the *sativum* band was present; for a *P. abyssinicum* marker, sum of those individuals that were resistant and the *abyssinicum* band was present. Number of recombinant individuals for a *P. sativum* marker, sum of those that were susceptible and the *sativum* band absent; for a *P. abyssinicum* marker, those that were resistant and the *abyssinicum* band absent. Recombinant frequency was calculated for each marker as the number of recombinants divided by the number of parental and recombinants. Linkage intensity was estimated considering a single group of markers, treating resistance as monogenic recessive.

The recombination fraction (r) is problematic to estimate in these segregants because of the mixture of generations and the pedigrees involved. However, the recombination values should lie somewhere between those estimated as the populations were either F2s or recombinant inbreds. Accordingly, r (in cM) and LOD (logarithm of the odds for linkage) were calculated using Mapmaker version 3.0 considering resistance as monogenic recessive. Values for F3/F4/F5 mixed population should lie in between F2 and RI values (Table 13).

Despite the difficulty in applying a simple model to the calculation of recombination frequencies, the estimates treating F2 and RI models as upper and lower bounds (Table 13) suggest that these six markers lie within *ca* 25 cM of the locus which is the major determinant of resistance. It is possible that some of the phenotypic scores are influenced by modifier loci or other, weaker, genetic determinants of resistance segregating in this progeny. Thus, some of the phenotypic scores may not accurately reflect the allelic state of the major genetic determinant, and thus the estimates of linkage distance are probably exaggerated.

Genetic mapping of *P. sativum* bands

Two of the *P. sativum* bands cosegregating with susceptibility have been mapped in previous RI populations. The TC(e) band (corresponding to Tps1/203-) was mapped in the RI population JI281 x JI399 and the AA(c) band (corresponding to Tps1/141-) in the RI population JI15 x JI399. The TC(f) band did not segregate in the previous RI populations and therefore no map position could be obtained (ie. TC(f) is present in JI15, JI281 and JI399). In both crosses the loci derive from an equivalent region of linkage group V between r and its nearer telomere (Fig. 4).

Unfortunately the recombination frequency (and chiasma number) is different for this chromosome / linkage group in these two crosses (Hall *et al.*,1997), so it is

difficult to estimate the distance between these two markers, but they are probably about 20 cM apart, which is consistent with the scale of distances estimated in Table 13. In turn, these data suggested that *r*(wrinkled seed), *pa* (foliage colour) or *det* (determinate growth) alleles may serve as useful classical markers for the introgression of this trait.

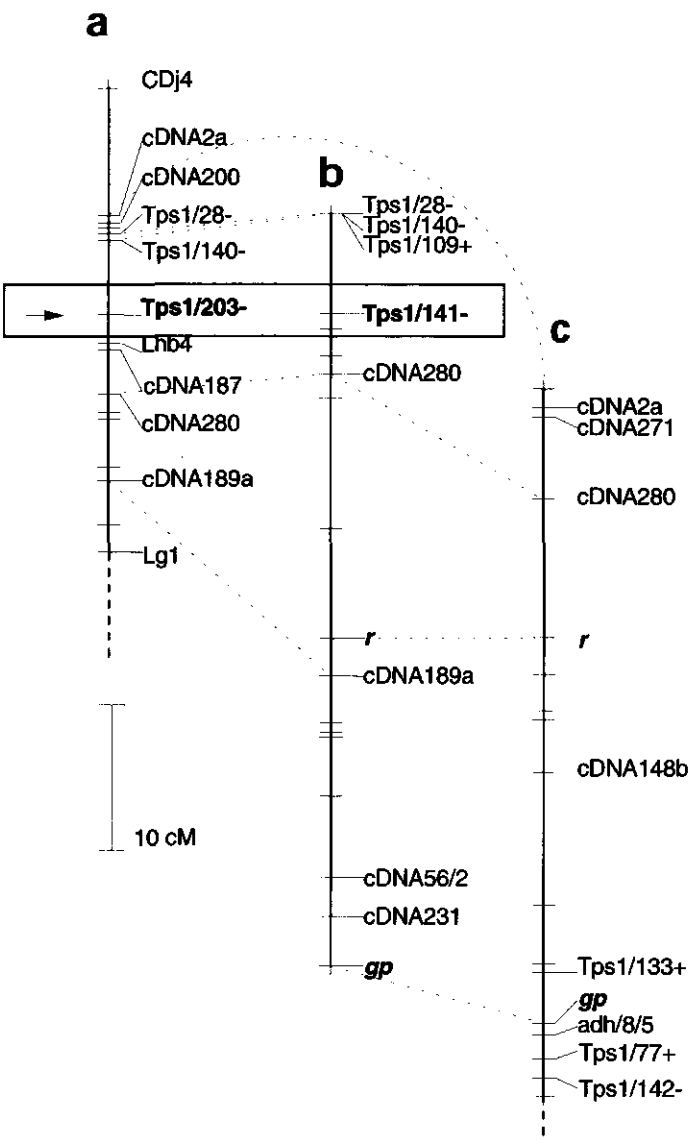


Fig. 4. Map of pea linkage group V derived from the recombinant inbred populations (a) JI281 x JI399, (b) JI15 x JI399 (b) and (c) JI15 x JI1194 showing the mapping position of the two identified markers cosegregating with susceptibility corresponding to Tps1/203- and Tps1/141- . Markers in common between the RI populations are connected by dashed lines

Table 13. Recombinant frequency (R) for the markers (from 'a' to 'f') for resistance identified with the selective bases TA, AT, AA and TC, recombination fraction (r) and LOD considering resistance as monogenic recessive with either an F2 or RI model for the population when calculated with Mapmaker version 3.0

	R	F2		RI	
		r	LOD	r	LOD
TC(f)	0.273	38.3	1.67	23.5	2.04
TC(e), Tps1/203-	0.186	24.9	2.59	13.0	3.96
AA(c), Tps1/141-	0.195	27.4	2.12	13.9	3.54
TA(a)	0.159	19.9	3.46	10.5	4.86
AT(b)	0.159	19.9	3.46	10.5	4.86
AA(d)	0.190	24.0	2.86	13.4	3.75

DISCUSSION

Genome compatibility

One of the possibilities to be considered to explain the complex F3 segregations is the relative incompatibility between the *P. sativum* and *P. abyssinicum* genomes. Karyotypic variability in pea can have an influence on segregation patterns (Ellis, 1993). Although crosses between *P. sativum* and *P. abyssinicum* were relatively successful, genetical and cytological analysis performed on these crosses suggest the presence of karyotypic differences between both genomes (von Rosen, 1944; Lamprecht, 1964; Saccardo, 1971; Conicella and Errico, 1985). The low and irregular seed setting at F1 is influenced by semi-sterility barriers (von Rosen, 1944; Conicella and Errico, 1990). *P. abyssinicum* has been reported as having 100.9-109.7 % of the genome size of *P. sativum* while other authors suggest that the genome size of *P. sativum* is practically constant (Baranyi *et al*, 1996; Baranyi and Greihulber, 1996). It is possible that there may be small local differences in repeated sequence organization between these two genomes, which may in turn contribute to unusual segregation patterns.

Maternal effect

Reciprocal differences in F1 fertility were observed, this was lower for crosses involving *P. abyssinicum* as the female parent. Only one cross with 'K. Wonder' (a

reciprocal involving JI2202) fitted neither the F2 ratio 1:3 R:S, when intermediate plants were incorporated in resistant category, nor the expected F2 ratio 3:1 T:D, this may be associated with the lower F1 fertility in this reciprocal cross. Von Rosen (1944) reported that crosses with *P. abyssinicum* as the female parent showed 8% fewer recessive individuals than the reciprocal. He referred to the difficulty of cooperation between genome and 'plasm' as the cause for this difference.

Imprinting effects, epigenetic modification of loci resulting in differential expression depending on parent of origin, could provide an explanation for differences in reciprocal crosses. Lund *et al.* (1995) found that some alleles of zein genes, which encode storage proteins, are only transcribed when maternally derived. Maternally and paternally derived genomes are not functionally equivalent in *Arabidopsis* endosperm (Scott *et al.*, 1998).

Apparent linkage of resistance to dwarfness

An apparent linkage of resistance to dwarfness was observed at F2, however, statistical analysis did not confirm this. Most of the F3 families falling in either 13:3 R:S category or uniformly resistant category were dwarf and a few segregated for height, but they none were uniformly tall. F5s, derived from resistant selections at F2 and F3, showing an apparent stabilized resistance were predominantly dwarf.

The association of resistance with dwarfness suggests a possible linkage between these characters. Nevertheless, a phenotypic association may not necessarily reflect genetic linkage, and a complete investigation of the inheritance of the dwarf character has not been undertaken. Von Rosen (1944) described the phenomenon 'varying linkage' caused by small structural changes in chromosomes.

Effect of race specific genes in combination with race non-specific resistance

In crosses with 'Fortune' the segregation at F2 showed an excess of resistant plants and there was generally a poor fit to 1:3 R:S ratio. It is thought that in this case resistance may have been enhanced by the background presence of the mainly dominant race specific genes (R2, R3 and R4) known to be present in 'Fortune'. This was also seen in subsequent generations. Only two families were found to be uniformly resistant at F3 (in crosses with Fortune), although the small number of plants in each progeny could not entirely preclude a hidden 13:3.

Additive effects of race specific genes and race non-specific have been previously reported (Taylor *et al.*, 1996). However, it is not really known how race-specific genes

to other races than race 6 of *Ps. syr.* pv. *pisi* can enhance the resistance to race 6 when they are present together with RNSR.

The identification of molecular markers for resistance with the PDR1-SSAP method

The PDR1-SSAP method proved to be a good marker system for studying segregating populations from crosses involving *P. sativum* and *P. abyssinicum*. Six markers for resistance were identified, three with *P. abyssinicum* origin and three with *P. sativum* origin. The fact that all the plants in which the three *P. sativum* bands were absent and the three *P. abyssinicum* bands were present, were resistant indicates that this set of six markers could assist in breeding programmes for selection of resistant plants. Of the resistant plants in which some of the *P. sativum* bands were present, half of them derived from 1:1 or 3:13 R:S F3 categories. This suggests that more than one loci may be involved in resistance.

Proposed models of inheritance

The segregation patterns at F2 suggested a recessive type of inheritance, however, relatively large numbers of individuals are needed to distinguish between one or two gene models. Specifically a 13:3 ratio is easily mistaken for 3:1 in small populations. The F3 ratios did not fit with a single recessive gene, however, despite the abnormal patterns, each of the observed ratios *per se* suggests that resistance is controlled by more than one gene but not by many. Another observation supporting this hypothesis is that if resistance was controlled by polygenes, the F2 would have shown a normal distribution with the majority of the F2 plants falling in the intermediate response category which was clearly not the case.

The difficulty arises in how to integrate in a model all the segregation ratios observed at F1, F2 and F3. We propose that resistance is controlled mainly by a recessive gene present in *P. abyssinicum* and a set of modifiers, most likely two. The interaction between the resistance gene and the modifiers is not known. The essential difference between *P. abyssinicum and P. sativum* consists of a difference in the allelic state of a small number of quantitative genes, these differences arose during the endemic period of development of *P. abyssinicum* (von Rosen,1944).

Our observations with RNSR in *P. abyssinicum* prompt comparison with the *mlo* gene for powdery mildew (*Erysiphe graminis* f. sp. *hordei*) resistance in barley. They both occur naturally in Ethiopia, confer a spectrum of quantitative resistance to all

races of the pathogen and have a recessive nature. The RNSR in barley was originally created by chemical mutagenesis (Freisleben and Lein, 1942) and only later found to occur naturally in Ethiopian barleys (Nover, 1968). Recently, through a mutation approach, Freialdenhoven *et al.* (1996) identified two modifiers required for the function of *mlo* and proposed a model in which the *Mlo* wild type allele functions as a negative regulator and the modifier genes (two unlinked recessive loci) act as positive regulators of a race-non specific response. Although the precise nature of the genetic control of RNSR in the *P. sativum* and *P. abyssinicum* crosses is unknown, it is possible that the presence of modifiers genes, derived from *P. sativum* or *P. abyssinicum* could explain at least some of the segregations in F3.

It would have been useful to compare the inheritance of RNSR in both *P. sativum* x *P. abyssinicum* and *P. abyssinicum* x *P. abyssinicum* crosses. At the time this work was initiated, all *P. abyssinicum* accessions appeared to carry RNSR. Only recently have we identified JI2385, which appears a single race specific gene but non RNSR. There is thus now a possibility to test the segregation of RNSR in its natural *P. abyssinicum* background.

The exploitation of race non-specific resistance derived from *P. abyssinicum*

The confusion that has hovered about the terminology and concepts concerning disease resistance in plants has been of enormous magnitude (Nelson, 1978). We use the term race non-specific resistance (RNSR) to define a rate-reducing resistance which occurs across all the races of the pathogen known at present, including race 6, for which there are no known resistant commercial cultivars. Also the fact that race 6 probably arose by the loss of an avirulence gene (from another race, either 2, 3 or 4) suggests that the resistance present in *P. abyssinicum* is race non-specific and that the genetic control involved is different from race specific resistance.

The motive for exploitation of RNSR is the need for durable resistance. In the case of pea bacterial blight, the need of RNSR became apparent with the existence of race 6 for which all tested cultivars to date are susceptible, and may cause severe losses particularly in winter sowing peas. Similarly, the need for durability in the control of powdery mildew was noticed for barley. Initially, breeders were reluctant to introduce the *mlo* gene in their breeding programmes mainly due to its pleiotropic effects (Kjær *et al.*, 1990). But due to the breakdown of race specific resistance, the use of the *mlo* gene was considered as one of the major strategies in the breeding for powdery mildew resistance. In spite of the fact that the great majority of research was done on mutagen-derived *mlo* resistance genes, it was three Ethiopian sources of *mlo* resistance that were first introduced in commercial varieties on the European market

(Jørgensen, 1992).

The RNSR present in *P. abyssinicum* opens new possibilities for the control of pea bacterial blight. First steps for the introduction of this resistance into important agronomical pea types are described in this paper. A first generation of crosses of F5 lines carrying race non-specifc resistance with commercial cultivars is available together with molecular markers to assist in the breeding programmes.

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ABSTRACT

Resistance to pea bacterial blight (*Pseudomonas syringae* pv. *pisi*) in different plant parts was investigated in twenty-one *Pisum sativum* commercial cultivars and landraces, carrying race specific resistance genes (R-genes) either singly or in various combinations; and two *Pisum abyssinicum* accessions carrying race non-specific resistance. The resistance genotype present in each pea accession had previously been determined by a standard stem inoculation procedure. Pea accessions were inoculated in stem, leaf and pod with seven races of *Ps. syr.* pv. *pisi* under glasshouse conditions. For both race specific and non-specific resistance, a resistant response in the stem was not always associated with resistance in leaf and pod. Race specific genes conferred stem resistance consistently, however, there was variability in leaf and pod responses. This depended on the matching combination R-gene/A-gene (avirulence gene). R2 generally conferred resistance in all plant parts, R3 or R4 singly did not generally confer resistance in leaf and pod, however R3 in combination with R4 had a positive additive effect and enhanced leaf and pod resistance. *P. abyssinicum* accessions were uniformly resistant in the stem to all races, showed leaf and pod resistance to races 2, 5 and 7 and variable , partial resistance or susceptibility, in leaf and pod to races 1, 3, 4 and 6.

The effects of environmental factors on disease expression were studied in the field under autumn/winter conditions. A universal susceptible cultivar (Kelvedon Wonder) and two *Pisum abyssinicum* accessions were inoculated with the most important races (races 2, 4 & 6). K. Wonder was susceptible to all three races but especially to race 4, the most aggressive race under field conditions. Eight weeks after inoculation all the K. Wonder plants were dead due to the combination of disease and frost damage. By comparison, *P. abyssinicum* was much less affected by disease, with only race 4 having a significant effect. The combination of disease resistance with frost tolerance in *Pisum abyssinicum* enabled plants to survive through the winter. It is suggested that a breeding strategy to combine race non-specific resistance with race specific resistance genes may provide durable resistance, under severe disease pressure, likely to be experienced in autumn/winter conditions.

INTRODUCTION

Studies on resistance to pea bacterial bight (*Pseudomonas syringae* pv. *pisi)* have been focused on race specific resistance. The presence of race specific resistance genes (R-genes) is widespread and at a high frequency both in commercial cultivars and *Pisum* germplasm. There are seven races of *Ps. syr.* pv. *pisi* currently recognized and interaction of races and cultivars is controlled by a gene-for-gene relationship (Taylor *et al.*, 1989; Bevan *et al.*, 1995).

Tests for resistance have usually been done by a stem inoculation procedure (Malik

et al., 1987). This method allows sequential multiple inoculations with different races on the same plant and avoids the risk of contamination associated with spray inoculation of leaves. It was thought that the response in the stem was likely to be the same for other plant parts. However, in stem inoculation with race 3, of a cultivar carrying the resistance gene R3, it was observed that when stem inoculation was very close to the stipule, sometimes a water-soaked susceptible response developed spreading into the stipule, while the stem showed a necrotic resistant response, indicating that stem and stipule (leaf) tissues may express a differential response to the pathogen.

There is some evidence in another host/pathogen combination (*Phaseolus vulgaris*/*Ps. syr.* pv. *phaseolicola* for differential responses in different plant parts (Hill *et al.*, 1972). In this case three different genes were reported to control separately the expression of halo blight in leaf, pod and systemic chlorosis.

In addition to the race specific genes in *Pisum*, there is a new identified class of resistance of a race non-specific nature derived from *Pisum abyssinicum* (Schmit *et al.*, 1993; Elvira-Recuenco and Taylor, 1998). This confers a quantitative stem resistance to all races of the pathogen including race 6 for which there are no known resistant cultivars. *P. abyssinicum* is tolerant to low temperatures and is characterized by its cosmopolitan qualities (Vavilov, 1992).

Moreover, the effects of environmental factors on disease expression are important in the epidemiology of pea blight. Frost damage increases susceptibility of plant tissue to the pathogen (Boelema, 1972). Disease severity is greater in winter sown than in spring sown peas for both winter and spring cultivars (Mansfield *et al.*, 1997). Factors that may be involved are high soil moisture contents which influences transmission of the pathogen from seed to seedling (Skoric, 1927; Roberts, 1992), frost and hail damage which favours entry of the pathogen (Young and Dye, 1970; Roberts *et al.*, 1995) and wind blown rain which favours dissemination of the pathogen (Stead and Pemberton, 1987).

Because of the possibility of differential responses between stem and leaf tissue, already demonstrated for race 3 with cultivars carrying R3, it was decided to investigate the full range of responses of both race specific and race non-specific resistance to all races of *Ps. syr.* pv. *pisi* in stems, leaves and pods under controlled glasshouse conditions. Investigations were also done in the field under autumn/winter conditions, when environmental factors (rain, frost, etc.) would be likely to predispose plants to maximum disease expression.

MATERIAL AND METHODS

Glasshouse studies

The pea lines and bacterial isolates used in this study are listed in Table 1. Peas were sown in compost in seed trays and grown at 20°C ± 2 day/17°C ± 2 night with supplementary lighting to give a 12 h day.

Bacterial isolates

Seven type strains corresponding to the seven races of *Ps. syr.* pv. *pisi* were used for inoculation. In some of the experiments an additional isolate of each race was used (Table 2).

For inoculum production, isolates were subcultured onto King's B medium (King *et al.*, 1954) plates for 24-48 h at 25°C. They were derived from one transfer on KB of cultures stored either at –80°C in broth (8g/l nutrient broth, 150ml/l glycerol) or freeze dried.

Stem inoculation

Pea seedlings were stem inoculated approximately two weeks after sowing (Malik *et al.*, 1987). Isolates were scraped from the surface of the plate with the tip of a sterile entomological mounting pin and stabbed into the main stem at its junction with the stipules at the youngest node.

Leaf inoculation

This was done simultaneously with stem inoculation. Young leaves were wounded with the same entomological pin used for stem inoculation and sprayed with a bacterial suspension (*ca* 10^9 cfu/ml) prepared in sterile ¼ strength Ringer's or sterile tap water. A wetting agent (Manoxol) was added to give a concentration of 0.025% in the bacterial suspensions which were sprayed at a low pressure to throughly wet the leaf surfaces. Inoculated plants were maintained in a mist chamber for 48 h after inoculation and then transferred to the glasshouse.

Pod inoculation

Young flat detached pods were placed on moist filter papers in Petri dishes. Isolates were scrapped from the surface of culture plates with sterile entomological pins and

pods stab inoculated (3-4 inoculations per pod). Petri dishes with pods were stored in closed boxes at room temperature.

Table 1. Pea accessions and their resistance characteristics to *Pseudomonas syringae* pv. *pisi*

Accession Number[a]	Designation	Stem resistance to races:	Resistance genes[b]
Pisum sativum cultivars			
JI2430	Kelvedon Wonder*	none	none
JI2431	Early Onward*	2	R2
JI2432	Belinda*	1, 3, 7	R3
JI2435	Hurst's Greenshaft*	1, 4, 5, 7	R4, (R6)
JI2438	Partridge*	1, 3, 4, 5, 7	R3, R4
JI2436	Vinco*	1, 2, 3, 5, 7	R1, R2, R3, (R5)
JI2437	Sleaford Triumph*	1, 2, 4, 5, 7	R2, R4, (R5)
JI2439	Fortune*	1, 2, 3, 4, 5, 7	R2, R3, R4
ZP-0034	Frisson	1, 2, 3, 5, 7	R2, R3
	Jade	1, 3, 7	R3
	Shasta	1, 3, 7	R3
	Martus	1, 3, 7	R3
Pisum sativum landraces			
JI171	*P. sativum* (Ethiopia)	1, 2, 3, 4, 5, 7	R2, R3, R4
JI1577	*P. sativum* (China)	1, 3, 4, 5, 7	R3, R4
ZP-0101	*P. sativum* (Spain)	1, 3, 4, 5, 7	R3, R4
ZP-0103	*P. sativum* (Spain)	1, 3, 7	R3
ZP-0104	*P. sativum* (Spain)	1, 2, 3, 4, 5, 7	R2, R3, R4
ZP-0107	*P. sativum* (Spain)	1, 3, 7	R3
ZP-0110	*P. sativum* (Spain)	1, 2 , 3, 4, 5, 7	R2, R3, R4
ZP-0112	*P. sativum* (Spain)	1, 3, 4, 5, 7	R3, R4
ZP-0137	*P. sativum* (Spain)	1, 3, 7	R3
Pisum abyssinicum			
JI1640	*P. abyssinicum* (Ehiopia)	1, 2, 3, 4, 5, 6, 7	Race non-specific
JI2202	*P. abyssinicum* (Ethiopia)	1, 2, 3, 4, 5, 6, 7	Race non-specific

* Differential cultivars (Bevan *et al.*, 1995); [a] JI numbers correspond to accession numbers in *Pisum* germplasm collection, John Innes Centre, Norwich, UK, ZP numbers correspond to accession numbers in the collection of 'Servicio de Investigación Agraria', 'Junta de Castilla y León', Valladolid, Spain; [b] Designation of resistance genes (Bevan *et al.*, 1995)

Table 2. Source and origin of the isolates of *Pseudomonas syringae* pv. *pisi*

Race	Isolate Number HRI-W	Source	Origin		
			Cultivar	Country	Year
1	299A*	ICPM 2955	Rondo	New Zealand	1970
1	1684	ICPM 3198	Unknown	India	1971
2	202*	ICPM 815	Unknown	U.S.A.	1944
2	4616	HRI-W	Solara	UK	1992
3	870A*	HRI-W	Martus (seed)	U.S.A.	1975
3	2191A	HRI-W	Rondo	Canada	1988
4	895A*	HRI-W	Martus (seed)	U.S.A.	1975
4	2817A	HRI-W	Unknown	Spain	1991
5	974B*	HRI-W	Puget (seed)	U.S.A.	1978
5	4012	HRI-W	Snowflake	Zimbabwe	1988
6	1704B*	MAFF	Stehgolt (seed)	France	1986
6	4129B	HRI-W	Stanton	UK	1990
7	2491A[a]*	ICPM 5316	Unknown	Australia	1976
7	4409	HRI-W	Bikini	UK	1991

HRI-W, Horticulture Research International, Wellesbourne, Warwickshire, U.K.

ICPM, International Collection of Micro-organisms from Plants, Plant Diseases Division, DSRI, Auckland, New Zealand

MAFF, Ministry of Agriculture, Fisheries and Food, Cambridge, U.K.

* Type strains

[a], Selection from ICPM 5316

Inoculation responses

Stem and leaf responses were recorded 7-10 days after inoculation and pod reactions 5-7 days after inoculation. Responses were assigned to one of three categories: a typical susceptible response showed an area of water-soaking spreading from the site of inoculation, whereas a typical resistant response resulted in necrosis localized at the point of inoculation. Incomplete expression of resistance was characterized by a combination of resistant and susceptible symptoms: localized necrosis surrounded by a limited water soaked area.

Winter outdoors studies

Cultivar K. Wonder (susceptible to all races) and *P. abyssinicum* accessions JI2202 and JI1640 (race non-specific resistance) were used for these studies. They were sown in compost in seed trays in a glasshouse. Three weeks later they were transplanted to 25 cm diameter pots containing John Innes compost number 1 and placed in a series of 'cold frames' with removable glass cover. Temperatures were expected to be similar to those in the open field but with some protection from the wind.

One week after transplanting the 'cold frames' were uncovered and two days later plants were either inoculated with the type strains of the races 2, 4 or 6 (Table 2) or uninoculated. Inoculation was done by spraying the plants with a bacterial suspension (*ca.* 10^9 cfu/ml) prepared as described above for glasshouse studies. Each treatment was represented by a block of 16 pots (4 pots per accession) distributed randomly. Treatments were separated by a minimum of 2.5 m. Disease symptoms and condition of the plants were recorded 1, 3, 5 and 8 weeks after inoculation. Meteorological data was also recorded.

RESULTS

Glasshouse studies

Race specific resistance

Stem inoculations with the seven races of *Ps. syr.* pv. *pisi* consistently gave the reactions expected from Bevan *et al.* (1995) (Table 3) and confirmed in the present study (Table 4). When the appropriate R-gene was present in the pea accession, the stem inoculated race carrying the matching avirulence gene caused a resistant (hypersensitive) response. The only exception was race 4 where some partial stem susceptibility was observed.

Inoculation responses in leaves and pods in some cases differed from those in the stems (Table 4). A susceptible response in the stem was always associated with a susceptible response in leaf and pod. However, a resistant response in the stem was associated with either a resistant or susceptible/partially susceptible response in leaf and pod. There was also some variation in inoculation responses between experiments and to some extent within the same experiment. In general responses in leaves and pods were similar although in some cases pods showed more susceptibility. Responses of the different host-race combinations (Resistance gene/Avirulence gene) were as follows:

Table 3. Gene-for-gene relationship between pea cultivars and races of *Pseudomonas syringae* pv. *pisi* (Bevan *et al.*, 1995)

							Race/avirulence genes						
							1	2	3	4	5	6	7
							1	.	.	.	.	.	.
							.	2	.	.	2	.	2
							3	.	3	.	.	.	3
							4	.	.	4	4	.	4
							.	.	.	.	(5)	.	.
	Resistance (R) genes						(6)	.	.	.	(6)	.	.
KelvedonWonder	.	.	.	.	.	.	+	+	+	+	+	+	+
Early Onward	.	2	.	.	.	.	+	-	+	+	-	+	-
Belinda	.	.	3	.	.	.	-	+	-	+	+	+	-
Hurst's Greenshaft	.	.	.	4	.	(6)	-	+	+	-	-	+	-
Partridge	.	.	3	4	.	.	-	+	-	-	-	+	-
Sleaford Triumph	.	2	.	4	(5)	.	-	-	+	-	-	+	-
Vinco	1	2	3	.	(5)	.	-	-	-	+	-	+	-
Fortune	.	2	3	4	.	.	-	-	-	-	-	+	-

+, Susceptible response to stem inoculation; -, resistant response to stem inoculation; genes in parentheses partly proven; ., gene absent

Race non-specific resistance

In *P. abyssinicum* accessions carrying race non-specific resistance (JI2202 and JI1640) challenged with races 2, 5 & 7, resistance expression was generally complete in stems, leaves and pods with the exception of JI1640 which showed partial resistance in the pod with race 2. Resistance to races 1, 3, 4 & 6 was complete in the stem but only partial in leaves and pods to races 1 & 6 and generally susceptible in leaves and pods to races 3 & 4.

Aggressiveness of isolates

Isolates of the same race gave the same responses with only a small variation in aggressiveness. Differences in aggressiveness between races were greater and race 4 was shown to be the most aggressive. Isolates 895 A, 1704B and particularly 870A were more aggressive in the leaf when they were derived from cultures preserved by freeze drying than those preserved at –80°C.

Table 4. Inoculation responses of pea accessions to seven races of *Pseudomonas syringae* pv. *pisi* in different plant parts (stems, leaves and pods)

Pea accession (Resistance gene)	Race	R/A[a]	Responses		
			Stem	Leaf	Pod
Early Onward (R2)	2	2	R	R	R
	5	2	R	R	R
	7	2	R	R/S	R/S
Belinda (R3)	1	3	R	R/S	R/S
	3	3	R	R/S	R/S
	7	3	R	R/S	R/S
Hurst's Greenshaft (R4+6)	1	4+6	R	R	R
	4	4	R	R/S	R/S
	5	4+6	R	R/S	R/S
	7	4	R	R/S	R/S
Partridge (R3+R4)	1	3+4	R	R	R
	3	3	R	R/S	R/S
	4	4	R	R/S	R/S
	5	4	R	R*	R*
	7	3+4	R	R*	R
Sleaford Triumph (R2+R4+R5)	1	4	R	R*	R/S
	2	2	R	R	R
	4	4	R	R/S	S
	5	2+4+5	R	R	R
	7	2+4	R	R	R/S
Vinco (R1+R2+R3+R5)	1	1+3	R	R	R
	2	2	R	R	R
	3	3	R	R	R/S
	5	2+5	R	R	R*
	7	2+3	R	R	R
Fortune (R2+R3+R4)	1	3+4	R	R	R
	2	2	R	R	R
	3	3	R	R/S	R/S
	4	4	R	R/S	S
	5	2+4	R	R	R
	7	2+3+4	R	R	R/S

Pea accession (Resistance gene)	Race	R/A	Responses		
			Stem	Leaf	Pod
Frisson (R2+R3)	1	3	R	R*	
	2	2	R	R	
	3	3	R	R/S	
	5	2	R	R	
	7	2+3	R	R	
JI171 (R2+R3+R4)	1	3+4	R	R	R
	2	2	R	R	R
	3	3	R	R*	R
	4	4	R	R/S	R/S
	5	2+4	R	R	R
	7	2+3+4	R	R	R
JI1577 (R3+R4)	1	3+4	R	R	R
	3	3	R	R/S	R/S
	4	4	R	R/S	S
	5	4	R	R/S	R/S
	7	3+4	R	R*	R*
ZP-0101 (R3+R4)	1	3+4	R	R	
	3	3	R	R/S	
	4	4	R	R/S	
	5	4	R	R	
	7	3+4	R	R	
ZP-0103 (R3)	1	3	R	R	
	3	3	R*	R/S	
	7	3	R	R/S	
ZP-0104 (R2+R3+R4)	1	3+4	R	R	
	2	2	R*	R/S	
	3	3	R	R*	
	4	4	R*	S	
	5	2+4	R	R	
	7	2+3+4	R	R	
ZP-0107 (R3)	1	3	R	R/S	
	3	3	R	R/S	
	7	3	R	R/S	

Pea accession (Resistance gene)	Race	R/A	Responses		
			Stem	Leaf	Pod
ZP-0110 (R2+R3+R4)	1	3+4	R	R	
	2	2	R	R/S	
	3	3	R	S*	
	4	4	R	S*	
	5	2+4	R	R*	
	7	2+3+4	R	R	
ZP-0112 (R3+R4)	1	3+4	R	R	
	3	3	R	R/S	
	4	4	R	S	
	5	4	R	R	
	7	3+4	R	R	
JI2202	1	rnsr[b]	R	R/S	R/S
	2	rnsr	R	R	R
	3	rnsr	R	S*	S
	4	rnsr	R	S*	S
	5	rsnr	R	R	R
	6	rsnr	R	R/S	R/S
	7	rsnr	R	R	R
JI1640	1	rnsr	R	R/S	R/S
	2	rsnr	R	R	R/S
	3	rsnr	R	R/S	R/S
	4	rsnr	R	S*	S
	5	rsnr	R	R	R
	6	rsnr	R	S*	R/S
	7	rsnr	R	R	R

R, uniformly resistant; S, uniformly susceptible; R/S, variable responses, partially resistant; R*, predominantly resistant responses; S*, predominantly susceptible responses; [a] R/A, resistance gene/avirulence genes, matching resistance and avirulence genes in pea accessions and *Ps. syr.* pv. *pisi* races; [b] race non-specific resistance

Winter outdoors studies

Symptoms were first observed in K. Wonder with race 4 only one week after inoculation (Table 5). Three weeks after inoculation disease development was moderately severe in K. Wonder inoculated with races 2 and 6 and very severe with race 4. By comparison *P. abyssinicum* accessions showed only a few small lesions with races 2 and 6 and slightly larger lesions with race 4 (Fig. 1). Eight weeks after inoculation, all the inoculated K. Wonder plants were dead whereas *P.abyssinicum* plants were alive and in generally good condition, although plants inoculated with race 4 showed some stem snapping. Uninoculated controls of both K. Wonder and *P. abyssinicum* showed no evidence of disease symptoms, and frost damage was more severe in K. Wonder than in *P. abyssinicum*. At least some of the *P. abyssinicum* plants survived through the winter to flower and produce seed.

Table 5. Disease development (D), frost damage (F) and survival (S) under winter conditions of the pea cultivar K. Wonder and the two *Pisum abyssinicum* accessions (JI2202 & JI1640) one, three, five and eight weeks after inoculation with *Pseudomonas syringae* pv. *pisi* races 2, 4 and 6

Treatment	1 w.	3 w.[a]		5 w.[b]		8 w.[c]		
	D	D	F	D	F	D	F	S
K. Wonder								
uninoculated	-	-	no	-	*	-	**	√
race 2	-	++		++		+++		no
race 4	(+)	+++		+++		+++		no
race 6	-	++		++		+++		no
P. abyssinicum								
uninoculated	-	-	no	-	no	-	*	√
race 2	-	(+)		(+)		+		√
race 4	-	+		+		++		√
race 6	-	(+)		(+)		+		√

[a] with 5 days frost; [b] with 10 days frost; [c] with 12 days frost; *, slight frost damage, **, severe frost damage; -, no symptoms; (+), few water-soaked lesions on lower leaves, mainly healthy; +, discrete water-soaked lesions on stipules; ++, fan shaped water-soaked lesions on stipules and leaves, moderately severe; +++, extensive fan shaped water-soaked lesions on stipules and leaves, severe

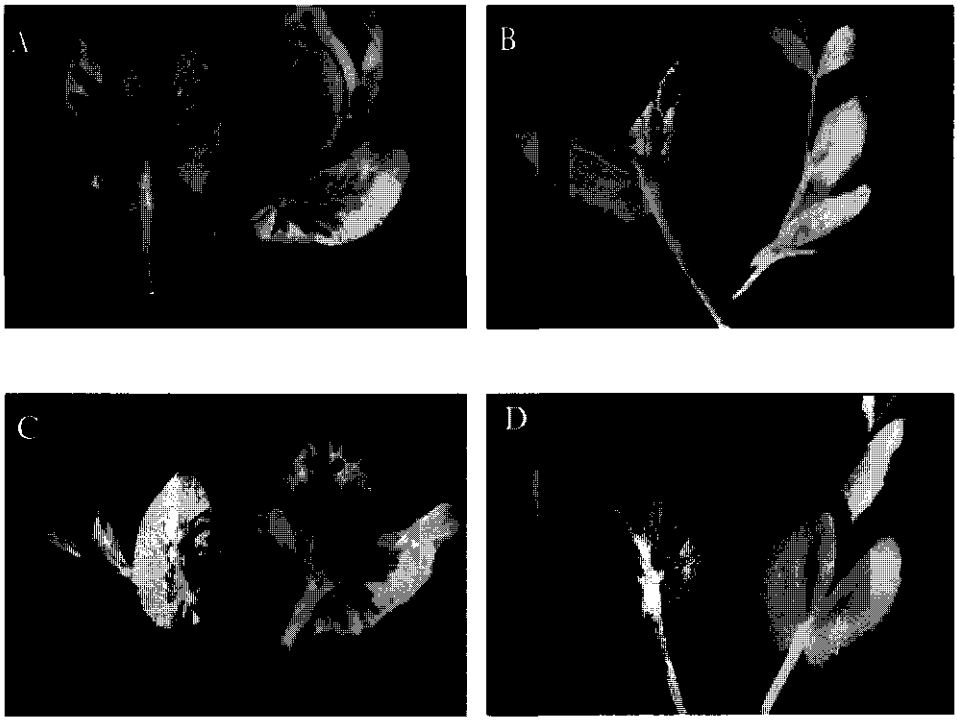

Fig. 1. Inoculation responses under autumn/winter conditions of (A) Kelvedon Wonder inoculated with race 4, (B) *Pisum abyssinicum* inoculated with race 4, (C) K. Wonder inoculated with race 6 and (D) *P. abyssinicum* inoculated with race 6

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Chapter 5

Natural incidence of endophytic bacteria in pea cultivars under field conditions

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ABSTRACT

Pea plants grown in the field were used to study the natural incidence of endophytic bacteria in the stem. Eleven pea cultivars at the flowering stage were screened for the presence of endophytic bacteria using a printing technique with surface disinfested stem cross-sections on 5% TSA. Five cultivars showed colonization of which cv. Twiggy showed the highest and most consistent colonization and was further investigated.

Stems of cv. Twiggy at the pod stage were analyzed for endophytic bacterial types and populations. Cross-sections from stem base to apex of surface disinfested stems were printed on 5% TSA. Endophytic bacterial populations decreased from the lower to the upper part of the stem. In addition, one section from the third and the fourth internode was surface disinfested, homogenized and spiral plated on three media: 5% TSA, R2A, and SC. Over a series of 30 samples, 5% TSA gave a significant 17% increase compared with R2A and SC. For most stems, populations ranged from 10^4 to 10^5 CFU/g except for one of the field blocks in which populations were uniformly higher. Comparison of colony counts by spiral plating and printing showed a positive correlation. Main bacterial types were *Pantoea agglomerans* and *Pseudomonas fluorescens.* Other frequent types were *Pseudomonas viridiflava* and *Bacillus megaterium.*

INTRODUCTION

Perotti used the term 'endophyte' in 1926 to describe the bacteria that had been isolated from within plants other than *Rhizobium* spp. Since then several definitions have been proposed for endophytic microorganisms (Chanway, 1996). Hallmann *et al.* (1997) described endophytes as bacteria which can be isolated from surface disinfested plant tissue or extracted from inside the plant and which do not visibly harm the plant.

The presence of endophytic bacteria in healthy plant tissue has been reported in many occasions for many plant species and plant parts at various stages of growth (Tervet and Hollis, 1948; Samish *et al.*, 1963; De Boer and Copeman, 1974; Misaghi and Dondelinger, 1990; McInroy and Kloepper, 1991). The genera most commonly isolated are *Pseudomonas*, *Erwinia*, *Bacillus*, *Burkholderia*, *Xanthomonas* and *Enterobacter* (Hallmann *et al.*, 1997).

Bacterial endophytes can have beneficial effects such as plant growth promotion and reduction of disease symptoms caused by plant pathogens (Chen *et al.*, 1995; Wei *et al.*, 1996; Sturz and Christie, 1997; Khmel *et al.*, 1998). However, in spite of lack of symptoms, endophytes may show negative effects on plant growth (Van Peer and Schippers, 1989).

For the pea crop, natural incidence of bacterial endophytes have only been investigated in ovules, seeds and pods (Samish *et al.*, 1963; Mundt and Hinkle, 1976). Nevertheless, there are reports on the potential induction of plant disease resistance by inoculated endophytes in pea root tissues (King and Parke, 1993; Castejón-Muñoz and Oyarzun, 1995; Benhamou *et al.*, 1996, 1998).

The aim of the present research was to study the incidence and major types of bacterial endophytes in the stem of various pea cultivars grown under field conditions. For this purpose different isolation procedures were used and evaluated.

MATERIAL AND METHODS

Screening of cultivars for the presence of endophytic bacteria

Collection of symptom-free pea plants was made in a breeding field trial of loamy soil with a randomized complete block design (five blocks), located in Haelen, Southern the Netherlands. Eleven cultivars, ten plants per cultivar (two plants per block), were sampled at the flowering stage. The cultivars tested were Bikini, Tristar, Geneva, Colana, Quattro, Twiggy, Sigra, Karisma, Nomad, Somerset and Hambado.

Stems were cut between the 2nd the 11th node and leaves were removed. Stem parts were surface disinfested with 1 % available chlorine (Presept tablets, Fisher Scientific) plus 0.1 % Tween 80 during 5 min. Then stems were rinsed three times in sterile distilled water. Cross-sections were made (Fig. 1) with an ethanol flamed scalpel. Per cross-section four prints were made on 5% Trypticase Soy Agar (TSA) with added 10 ppm 2,3,5-triphenyltetrazolium chloride (TTC) for colony differentiation and 100 ppm cycloheximide. Plates with prints were allowed to dry under a hood for 30 min and then the print area was covered with a 200 µl droplet of liquefied 5 % TSA at a temperature of 48° C to restrict colony size. Plates were incubated at 27° C during up to 10 days. Prints were recorded for bacterial growth and main types subcultured on 5% TSA for further characterization.

Differences between cultivars in endophytic colonization were analyzed by fitting a logistic regression model, with cultivar as variable, to the observed number of plants in which endophytic colonization was observed, using the method of maximum likelihood (McCullagh and Nelder, 1989).

Endophytic bacterial populations in the cultivar Twiggy

Twenty plants of the cv. Twiggy were sampled at the pod stage, collected from four

field blocks (five plants per block). Leaves were removed and the stem was cut in two parts, the lower and the upper part (Fig. 1). Both stem parts were surface disinfested as described above and then rinsed in sterile distilled water. The last rinse for the lower part was made in ¼ strength Ringers and 0.5 ml was plated on 5% TSA + 100 ppm cycloheximide for sterility checks.

Cross-sections (Fig. 1 and 2) were made as described above and prints from eight plants were covered with a layer of the same medium while the rest of the prints from 12 plants were not covered. Plates were incubated at 27° C and bacterial growth was recorded after 48 h and one week.

Third and fourth internodes were weighed and homogenized separately in ¼ strength Ringers using autoclaved mortars and pestles. Extracts were spiral plated (WASP, Don Whitley Scientific) on 5% TSA, R2A (Difco) and SC (Davis et al. 1980) with 100 ppm cycloheximide added to each of the three media and incubated at 27 °C. Sterility check plates were incubated for one week. Total numbers and numbers of dominant endophytic types were determined per spiral plate and calculated as number per gram fresh stem weight (Fig. 2).

The logarithmic transformed CFU/g values were analyzed by ANOVA using a splitplot model where effects of stem part, medium and interaction between stem part and medium were taken fixed. Additional random effects of block, plant and stem part were taken to describe interactions between the observations. In addition log CFU/g values averaged over both stem parts for the medium 5% TSA were analyzed by ANOVA .

The relationship between counts in prints and in spiral plating was approximated by the linear function $Y = \bullet + \bullet X$ where Y = log CFU/g plating (average of log CFU/g stem weight of internodes 3 and 4 when spiral plated) and X = CFU print (total number of colonies of stem transections 1, 2, 3, 4 and 5 corresponding to internodes 3 and 4 when agar printed) or X = log CFU print. Visual inspection of the appropriateness of the linear model were done from plots of log CFU/g plating against CFU print or against log CFU print. Based on these findings a linear regression of log CFU/ g plating on CFU print and 95% prediction intervals were performed.

Presence of bacteria inside the seed as source for endophytic colonization was investigated for cv. Twiggy. Twenty five seeds were surface disinfested by immersion in 2.5 % available chlorine suspension (Presept tablets, Fisher Scientific) plus Tween 20 during 5 min. and rinse with sterile tap water three times. Then they were dry ground and 1 ml of ¼ strength Ringers was added. This extract (100 µl per seed sample) was plated on 5% TSA and plates incubated at 27° C.

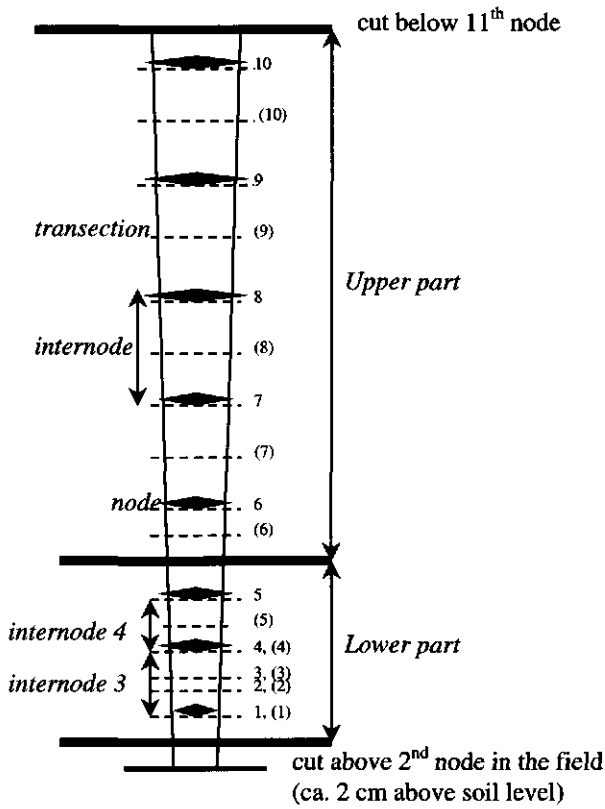

Fig. 1. Scheme of stem showing cross-sections printed on 5% TSA for the screening of cultivars (from 1 to 10 in parentheses), and cross-sections printed on 5% TSA (from 1 to 10) plus internodes 3 and 4 spiral plated for the cv. Twiggy screening

RESULTS

Screening of cultivars for the presence of endophytic bacteria

The printing of stem transections was applied as a fast screening and gave a semi-quantitative indication of the population levels. It was also possible to confirm the endophytic presence of the bacteria in the prints. Five cultivars showed clear endophytic colonization (Table 1), though Twiggy was the most consistently

colonized. The other five cultivars did not show any bacterial growth on the print, though they may contain populations below 3-4 log CFU/g fresh weight, the threshold of the printing technique based on later comparisons between printing and spiral plating.

For cv's Colana, Bikini, Sigra and Tristar prints of the 3rd or 4th internode developed bacterial growth, whereas for Twiggy there was growth on prints up to the 7th internode. Moreover the number of plants showing bacterial colonization was higher in Twiggy than in the rest.

Under the assumption of binomial variance, the P values of the • ^{2} test gave evidence for differences between cultivars (P<0.01). Based on the P values of the • ^{2} test for pairwise comparisons, the cultivars with no print-detected endophytic colonization (Geneva, Karisma, Nomad, Somerset and Hambado) differed from Twiggy (P<0.01). Furthermore, cultivars with only 10% of the plants colonized (Bikini, Sigra and Tristar) also differed from Twiggy (P= 0.04).

Table 1. Number of pea plants per cultivar showing bacterial endophytic colonization when using a printing technique for screening of stem cross-sections on 5% TSA

Cultivar	No. of plants[a]	Internode	Isolated types[b]
Twiggy	5	3, 4, 5, 6, 7	*Ralstonia pickettii* like
Colana	2	3	
Bikini	1	3	*Bacillus* spp.
Sigra	1	4	*Pantoea agglomerans*, *Ps. fluorescens* type
Tristar	1	3	*Rahnella aquatilis*, *Ps. fluorescens* type

[a] Number of plants out of 10 tested with colonized stem prints

[b] Identification by fatty acid profiling/BIOLOG of some isolated types

Endophytic colonization of the cultivar Twiggy

Stem extracts by spiral plating

Comparison of media

Table 2 presents the data for the field block 2 as an example to compare data on media. Mean values of populations for the three media gave differences less than 1 log CFU/g fresh weight for internode 3 and 4. Range of maximum and minimum

populations per sample for the three media was less than 0.50 log CFU/g fresh stem.

The means of log CFU/g over a series of 30 stem extracts of 3 blocks were 4.80 for 5% TSA, 4.73 for R2A and 4.73 for SC with SED=0.0264. The increase of 0.07 in log counts for 5% TSA corresponds with 17 % higher counts compared to those for R2A and SC. The ANOVA analysis of log CFU/g showed a significant effect of the agar medium (P=0.015). Pairwise comparisons of treatment means using t-tests showed that the mean for 5% TSA is significantly higher than those for R2A and SC, which do not differ significantly.

Table 2. Comparison of the numbers of endophytic bacteria in internodes 3 and 4 of the pea cv. Twiggy and isolation efficiency for three media when stem extracts were spiral plated on 5% TSA, R2A and SC. Data are presented for the field block number 2

Stem Section[a]	5% TSA	R2A	SC	Average	Range (max-min)
6a	4.64[b]	4.61	4.52	4.59	0.12
6b	4.32	4.04	4.23	4.20	0.28
7a	4.90	4.63	4.86	4.80	0.27
7b	4.98	4.48	4.56	4.67	0.50
8a	6.04	5.73	5.91	5.90	0.31
8b	5.32	5.26	5.41	5.33	0.15
9a	4.15	3.89	3.77	3.93	0.38
9b	3.80	3.99	3.86	3.88	0.19
10a	4.73	4.76	4.68	4.73	0.08
10b	4.89	4.90	4.97	4.92	0.08

[a] Code indicates plant number and stem part (a for internode 3, b for internode 4)

[b] Bacterial endophytic populations are expressed in log (colony forming units per gram fresh stem tissue)

Bacterial endophyte populations

Populations in the third and fourth internode within a plant were of the same order or one order difference. The populations at the 3rd and 4th internode of the stem ranged from 10^3 to 10^7 CFU/g, being usually between 10^4 to 10^6 CFU/g fresh weight (Table

3). Plants from the field block number 4 showed higher populations than the other blocks (10^6-10^7 CFU/g fresh weight). ANOVA on log CFU/g values on 5% TSA averaged over both stem parts with block as explanatory variable showed a significant effect of blocks ($P<0.001$). Pairwise comparisons of block means using t-test showed that the mean of block 4 is significantly larger than those of the remaining blocks, which do not differ significantly.

Sterility checks from 3 of the 20 samples were positive (ca. 10-10^2 CFU/g fresh weight), however generally the colony types present were phenotypically different from the types present in the stem extracts on 5% TSA.

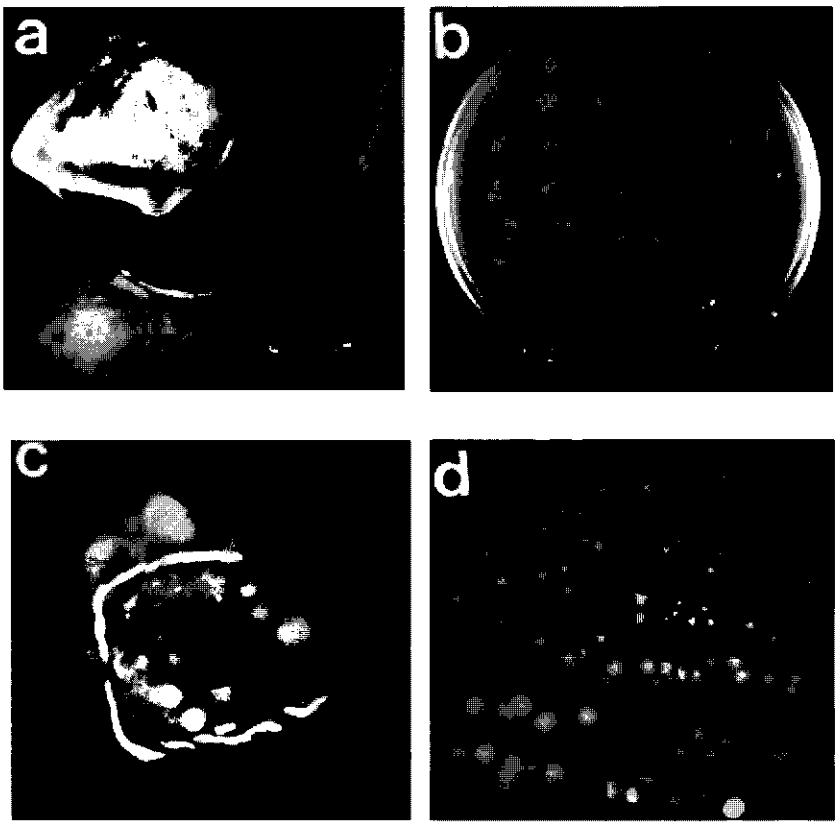

Fig. 2 a) Stem transections used for printing, node and internode (with cavity), b) Prints on 5% TSA of transections from the stem base to the tip (from right top to left bottom of the plate), c) Bacterial colony pattern in the print, d) Detail of a spiral plated stem extract showing some dominant bacterial types

Table 3. Total populations (CFU/g fresh stem weight) of endophytic bacteria and populations of *Pantoea agglomerans*, *Pseudomonas fluorescens*, *Pseudomonas viridiflava* and *Bacillus megaterium* for cv. Twiggy when stem extracts were spiral plated on 5% TSA

Plant No	Total Counts**	*Pantoea agglomerans*	*Pseudomonas fluorescens*	*Pseudomonas viridiflava*	*Bacillus megaterium*
Field Block 1					
1	4.85	4.82	3.74		
2	5.27	4.71*	4.38		
3	4.27	4.11	3.60		
4	5.93	5.33	4.46*	5.66	
5	4.85	4.79	3.78		
Field Block 2					
6	4.48	4.29	3.78		
7	4.94	4.59	4.13	4.52	
8	5.68	5.57	4.72	5.04*	
9	3.97	3.76			
10	4.81	4.65	4.56*	3.48*	
Field Block 3					
11	4.56	4.50	3.58		
12	4.51	4.38			
13	4.49	3.99	4.24		
14	4.21	3.97			
15	5.11	4.84	4.76		
Field Block 4					
16	6.69	6.44	6.23		4.44
17	6.22	6.04	5.48	5.32*	4.91
18	6.75	6.11	6.17	6.44	5.20*
19	7.16	7.03	6.28	6.20	4.55
20	6.94	6.79	6.04	6.20	4.85*

*only present in one of the internodes

** Average of log CFU/g for internodes 3 and 4

Stem prints

Prints of cross-sections proved to be an efficient tool for detection and semi-quantitative estimation of endophytic bacteria (Table 4).

Prints covered with a second layer of 5% TSA reduced colony size and kept colonies separated in highly colonized areas. This allowed for some samples a better isolation of types from densily colonized prints. However, part of the bacteria spreaded outside the print area which made interpretation more difficult.

Density in the prints of different stem parts of the same Twiggy plant was usually higher in the lower part of the stem than in the upper part. Endophyte colonies were present in prints of the 3rd and 4th internode for all the plants, and very limited or nil in prints of the 7th-9th internodes (Table 4).

The observed and fitted linear relationship between counts in prints and CFU/g by spiral plating are plotted in Fig 3. as well as a 95% prediction interval. Printing seems to be a good method for semi-quantitative estimation of populations in stems containing 10^4 to 10^7 CFU/g fresh weight. There is variation of points around the fitted line and predicted counts of endophytic populations using the printing technique are expected to be more accurate for high populations.

Fig. 3 Relationship between observed values of the average of log CFU/g fresh stem of internodes 3 and 4 when spiral plated and the total number of colonies of stem prints corresponding to internodes 3 and 4

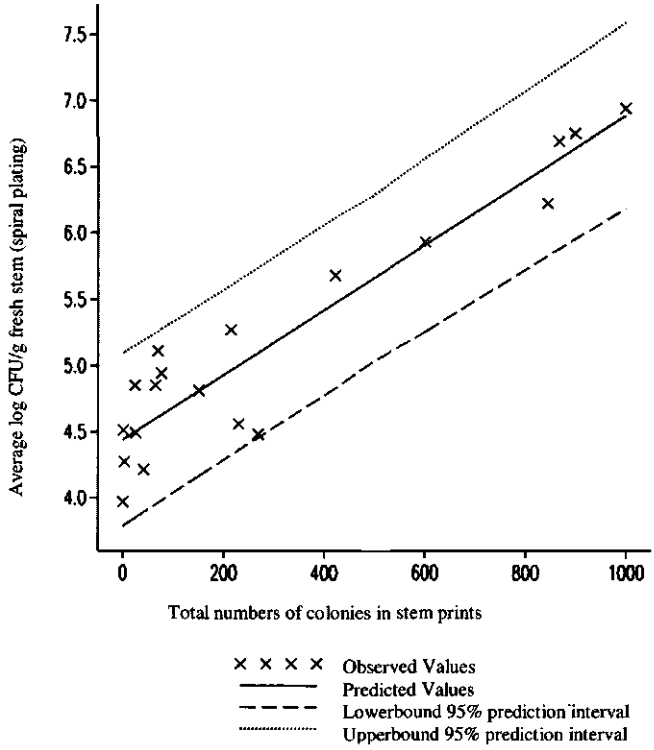

Table 4. Number of endophytic bacterial colonies per stem cross-section of cv. Twiggy when printed on 5%TSA

Plant/	Transection Number[a]									
Block	1	2	3	4	5	6	7	8	9	10
1/1	29[b]	8	16	1	11	0	0	0	0	0
2/1	0	2	3	200	0	0	0	0	0	0
3/1	2	0	1	0	0	0	5	0	0	0
4/1	197	28	100	76	>200	43	0	0	0	0
5/1	5	0	0	9	10	0	4	3	0	0
6/2	>200	26	30	1	12	0	1	0	0	1
7/2	5	5	53	5	9	0	0	0	0	0
8/2	>200	24	84	100	14	8	1	1	29	0
9/2	0	0	0	0	0	12	8	0	0	0
10/2	37	18	0	42	54	0	0	15	0	0
11/3	>200	5	0	7	18	2	4	1	1	0
12/3	1	0	0	0	0	15	0	0	0	0
13/3	14	0	5	6	0	>200	8	20	0	0
14/3	10	4	21	6	0	0	15	1	8	0
15/3	7	6	26	31	0	38	0	0	0	0
16/4	>200	>200	>200	67	>200	>200	>200	5	0	0
17/4	>200	>200	>200	>200	44	>200	4	0	0	0
18/4	>200	>200	>200	>200	>200	>200	8	>200	0	0
19/4	>200	>200	44	1	49	17	0	0	0	0
20/4	>200	>200	>200	>200	>200	>200	>200	>200	>200	>200

[a] Cross-section number 1,2,3 corresponds to internode 3. Cross-sections 4,5 to internode 4. Cross-sections from 6 to 10 to internodes 6, 7, 8, 9 and 10

[b] Total number of colonies per cross-section was determined by adding the number of colonies of the four prints made per cross-section

Seed tests

A total of 25 Twiggy seeds from the seed lot used in the field trials were analyzed for the presence of bacteria as potential source for endophytic colonization. Only two seeds showed some bacterial colonization at a level of 10^3 CFU/ml pea seed extract. The dominant bacterial type for each of these seeds could not be identified by the fatty acid profiling.

Bacterial characterization

Twenty five isolates representing dominant bacterial types, mainly from cv. Twiggy, were identified by fatty acid profiling and some isolates were also identified by BIOLOG (Table 5). The main bacterial types found were *Pantoea agglomerans* (*Erwinia herbicola*) being identified in all of the 20 plants studied and *Pseudomonas fluorescens* in 17 plants. Another types were *Pseudomonas viridiflava* (mainly in block 4), in 8 plants, *Bacillus megaterium* in 5 plants (only found in block 4), and others not identified (Tables 3 and 5).

Eight *Pseudomonas fluorescens* isolates (S321, S326, S327, S331, S332, S333, S334 and S335, IPO Bacteriology collection, Wageningen, the Netherlands) isolated from the stem of cv. Twiggy and two *P. fluorescens* isolates (S317, S318) from the root of cv. Tristar were analyzed by fatty acid profiling and classified in four different subgroups: (1) S321, S333, S334 and S335, (2) S326 and S332, (3) S318, S327 and S331 and (4) S317, the most distinct isolate.

Table 5. Species identification of some isolated bacterial endophytes from the stem of cv.Twiggy by Fatty Acid profiling and BIOLOG

Strain number[a]	Fatty acid profiling		BIOLOG	
	Identification	SI[b]	Identification	SI
S320	*Pantoea agglomerans*	0.886	*Pa. agglomerans*	0.909
S321	*Pseudomonas fluorescens* B	0.681	*Ps. fluorescens* G	0.778
S322	*Pseudomonas viridiflava*	0.961	*Ps. viridiflava* B	0.805
S325	*Pantoea agglomerans*	0.859	*Pa. agglomerans*	0.851
S326	*Pseudomonas fluorescens* B	0.758	*Ps. fluorescens* B	0.576
S329	*Pseudomonas viridiflava*	0.948	*Ps. syringae* pv. *aptata*	0.551
S331	*Pseudomonas fluorescens*	0.429	No identification	
S332	*Pseudomonas fluorescens B*	0.443	*Ps. corrugata*	0.565

[a] Strain number in collection at Bacteriology, IPO, Wageningen, the Netherlands, [b] Similarity index

DISCUSSION

Comparison of techniques for isolation of endophytic bacteria

Methods for examining bacterial endophytes have been focused on detection of

culturable endophytes by dilution plating for their simplicity and sensitivity compared with other methods as viable staining or electron microscopy. Nevertheless, two major limitations must be considered: (1) the surface disinfestation process is a compromise between elimination of the epiphytic population and maintenance of the endophytic population, (2) the culture media which will select bacteria favoured by the nutrients present in a certain medium (Bacon and Hinton, 1997). For this study three media described by McInroy and Kloepper (1995) were used. However, we did not use full strength TSA but 5% TSA as recommended by J.W. Kloepper (personal communication). Populations from the medium 5% TSA were significantly higher than populations on R2A and SC. McInroy and Kloepper (1995) found significantly greater populations on R2A and SC medium than on full TSA. This indicates that 5% TSA is probably a good general medium for isolation of bacterial endophytes.

Printing of cross-sections on 5% TSA (van Vuurde *et al.*, 1996) showed to be a good routine method to screen for the presence of bacterial endophytes in the pea stem and estimate populations between 10^4 to 10^7 CFU/g fresh weight. For populations below 10^4 CFU/g, prints are usually negative. In the print, endophyte cells from the stem tissue may be strongly clustered and result in lower number of colonies.

Endophytic colonization

Results in the screening of eleven cultivars using the printing technique on agar showed that the cv. Twiggy had significant higher bacterial colonization than the other cultivars tested. These results indicate that cultivar type plays a role in the efficiency of bacterial endophyte colonization. Adams and Kloepper (1998) suggested that colonization of cotton plants by endophytic bacteria is affected by plant genotype. Smith *et al.* (1999) found a genetic basis for the interactions between a recombinant inbred line population of tomato and root colonization by *Bacillus cereus*.

For the cv. Twiggy screening, populations at the stem base (3^{rd}-4^{th} internode) varied mainly between 10^4 to 10^6 CFU/g as reported in stems for other crops such as potato (De Boer and Copeman, 1974), alfalfa (Gagné *et al.*, 1987), cotton and sweet corn (McInroy and Kloepper, 1995). In field block 4, populations were significantly higher and ranged from 10^6-10^7 CFU/g for all the plants tested. It seems that certain conditions in that part of the field were more favourable for bacterial colonization of the stem. Both biotic damage, eg. due to a local high density of nematodes or plant invading fungi, and less likely, abiotic factors (local soil conditions) may explain this phenomenon.

Higher populations were observed in the lower part of the stem than in the upper part. The strong decrease of bacterial endophyte populations acropetally in the stem

has been also reported by Fisher *et al.* (1992). This indicates the seed and the soil as important initial sources of bacterial endophytes as reported by Misaghi and Donndelinger (1990), McInroy and Kloepper (1995) and Mahaffee and Kloepper (1997). The absence of the main endophyte types in the seed of cv. Twiggy points to the rhizosphere soil as the dominant source of the endophytes present in Twiggy.

Bacterial endophyte types

The main bacterial genera found for the cv. Twiggy were *Pseudomonas, Pantoea and Bacillus*. Identification at species level by fatty acid profiling and Biolog showed a good correlation between both methods except for some *Pseudomonas fluorescens* strains. Previous studies on pea seeds and pods (Samish *et al.*, 1963) and pea seeds and ovules (Mundt and Hinkle, 1976) also reported these genera as main bacterial endophytes. These types are commonly found as plant and soil bacteria (Hallmann *et al.*, 1997). *Pseudomonas* and *Bacillus* spp. have been reported as biocontrol agents of plant diseases (Cook *et al.*, 1996). The present data provide a basis for studies on microbial harmonization of pea production systems.

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Chapter 6

Effects of soil, plant genotype and growth stage on endophytic bacterial colonization of pea roots and stems in the field

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ABSTRACT

The effects of soil type, plant genotype, growth stage of the crop and plant part on endophytic bacterial colonization were studied in five pea genotypes: four pea (*Pisum sativum*) cultivars and one *Pisum abyssinicum* accession, at three field sites in the central-east of the Netherlands with different soil characteristics (sandy, light-clay and heavy-clay). Plants were sampled at the seedling and the flower stages and processed either pooled or individually. One root and one stem base section 2-3 cm long were sampled for young plants and an additional section of the middle part of the stem for flowering plants. Sections were surface disinfested, homogenized and extracts plated on 5% TSBA plates. Characterization of the main endophyte groups was made by the BIOLOG system and by the fatty acid profiling. At the seedling stage, there were significant differences according to soil type, pea genotype and plant part (root-stem base). At the flower stage, interactions between the main factors were detected. Significant differences between pea genotypes were only found for the root part. Significant differences were observed between the root or stem base compared with the middle stem. Light-clay soil gave the highest endophytic colonization for both growth stages, however, ranking of pea genotypes was not the same. There was an increase of the mean log counts at the flower stage (log 5.6 CFU/g) compared to the seedling stage (log 5.1 CFU/g). Gram-negative bacteria represented 90% of the total bacterial population: *Pseudomonas* sp. (54%), *Pantoea agglomerans* (11%) and *Stenotrophomonas maltophilia* (8%). *Arthrobacter* sp. and *Curtobacterium* sp. were the main Gram-positive species (4.2% and 1.6% respectively). There was an increase in the Gram-positive population up to 10 times higher in the mature plants. Specificity for plant part and/or growth stage was observed for *Ps. corrugata, Ps. viridiflava, Ps. syringae* pv. *syringae, Arthrobacter* sp., *Acinetobacter* sp. and *Agrobacterium* sp.

INTRODUCTION

Research on the ability of bacteria to colonize the interior of plants without causing visual symptoms started with the work of Pasteur on grapevine in 1876. Hollis (1951) reviewed the early bacterial endophyte literature showing the different views of the scientists on the natural existence of endophytic bacteria other than *Rhizobium* sp. Several examples of effective colonization of crop plants were given in the review of Hallmann *et al.* (1997). This review also highlights the large biodiversity across bacterial endophytes isolates, as found for example in cotton and corn (McInroy and Kloepper, 1995*a*) and grapevine (Bell *et al.*, 1995).

In general the presence of endophytic bacteria is expected to have a negative effect on plant growth, through competition for water and nutrients. However, the literature of the last decade demonstrates that the overall effect of certain endophytes isolates

can be highly beneficial for plant growth. Growth stimulation is the result of either an improved availability of nutrients as N, P and Fe through endophyte activity or directly through the formation of plant hormones (Sturz *et al.*, 2000). Improved plant growth can also be the result of a better protection of the plant against pathogens and pests after the introduction of selected endophytes. The net effect of natural endophytes on growth of individual plants in a crop seems to be related to the dominance of beneficial or deleterious bacteria in these plants.

Microbial harmonization of plants to optimize crop production and to reduce agrochemicals use is increasingly seen as an important tool in the development of sustainable plant production systems. Symbiotic endophytic bacteria are a compatible component of this approach as the niches colonized in the plant are rather stable and protected from environmental changes and microbial competition (Hallmann *et al.*, 1997).

The effects of environmental and cultural factors on the natural endophytic colonization of plants in production systems have hardly been investigated. Understanding of the biology of natural endophytes and their ecological behaviour in major crop systems will be essential for the development of an effective endophyte-plant management strategy.

In an earlier study on the natural incidence of endophytic bacteria in pea we have reported on differences in endophytic colonization of stems of pea plants at the pod stage for eleven cultivars (Elvira-Recuenco and Van Vuurde, Chapter 5). The aim of this study was to investigate possible differences in the endophytic colonization of four *Pisum sativum* cultivars and one *Pisum abyssinicum* accession grown in three different soils. This study was performed for young and mature plants up to the flower stage. Both colonizations of root and of stem parts were studied for total endophyte numbers and biodiversity.

MATERIAL AND METHODS

Plant material

Five pea genotypes, four *Pisum sativum* commercial cultivars and one *Pisum abyssinicum* accession (JI2202, John Innes Centre, Norwich, UK) were used for the studies on endophytic bacterial colonization. The cultivars were Twiggy, semi-leafless, a combining pea for animal feed; Solara, a combining pea for animal feed; Norli, a sugar snap pea (immature pod for human consumption) and Fortune, a vining pea (immature seed for human consumption).

Growth conditions and experimental design

Studies were performed at three sites in the eastern part of The Netherlands (Wageningen and Lienden) with different soil types: location De Eng (EN, sand, pH=7.6, organic matter=3.4%), location Schuilenberg (SC, light-clay, pH=7.2, organic matter=2.7%, clay=31-39%) and location Grebbedijk (GR, heavy-clay, pH=7.3, organic matter=4.0%, clay=42-51%). Sowing was done at the beginning of May, 1998, at a density of *ca* 80 plants/m^2. One kg of fertilizer was added in every location (12-10-18, N-P-K).

At each location, there were two fields of 52 m^2 (13m x 4m), with two blocks per field and five plots per block corresponding to the five pea genotypes. Plot areas were 3 m^2. Genotypes were distributed in plots according to a randomized complete block design.

Field sampling

Plants were taken from the field on two sampling dates (seedling and flower stage) and stored at 4°C. Five plants per plot (genotype) in block 1 of each field and two plants per plot in block 2 were sampled.

Sample processing

For seedlings, a 2-3 cm longitudinal root section from the seed downward and a 2-3 cm longitudinal stem base section from the seed upward were cut with a blade. For mature plants, an additional internode stem section was cut in the middle of the stem. For each plot, root and stem sections of the five plants from block 1 were pooled while sections of the two plants from block 2 were kept separately.

Sections were weighed and placed in 10 ml tubes for surface disinfestation with 1% available chlorine (Presept tablets, Fisher Scientific) plus 0.1 % Tween 80 for 5 min (seedling sections) and 5-10 min (mature plant sections). They were then washed twice with sterile distilled water and a third time with 10 ml ¼ strength Thiosulfate Ringer's to inactivate remains of chlorine. As a sterility check, 100 µl of the last rinse was plated on 5% TSBA + 100 ppm cycloheximide plates and incubated at 27°C for one week.

Surface disinfested sections were homogenized using a Kleco homogenizer (Kinetic Laboratory Equipment CO) in 10 ml of ¼ strength Thisosulfate Ringer's for pooled samples and in 5 ml for individual samples. Dilutions (100 times fold) of

extracts were prepared in ¼ strength Ringer's. Extracts and dilutions were plated (50 µl per plate) with a spiral plater (Don Whitley Scientific LTD) on 5% TSBA + 100 ppm cycloheximide plates and incubated at 27°C. For each sample plate, the total number of colonies and the number of colonies of the main bacterial types were counted and values of CFU/g fresh weight were calculated. Isolation of main types was done in 5% TSBA and isolates were stored at –80 °C for further characterization.

Statistical analyisis of bacterial counts

The observed bacterial counts (CFU/g) were analyzed after logarithmic transformation. A splitplot model with location (soil type), pea genotype, plant part and the two and three factor-interactions between these factors as explanatory variables was performed. Additional random effects to describe dependencies between observations were allowed for the model: for pooled samples random effects were allowed for differences between fields within location and plots within fields; for individual samples the same additional random effects were taken as well as random effects for differences between plants within plots.

The REML (restricted maximum likelihood) routine of the program Genstat 5 (1993) was used to estimate the various variance components and fixed effects in the model. A full model with fixed effects (location, pea genotype and plant part) and all interactions was fitted to he data. Data are presented for a smaller model in which effects of location, genotype, plant part and significant interactions are included. Fixed effects were screened by applying Wald-tests (Cox and Hinkley, 1974). Wald-statistics were calculated for the values of the variance components obtained for the corresponding model. Chi-square tests for the Wald-statistics were used ignoring variability due to estimation of the variance components. Pairwise differences between treatment means were tested using normal tests. Significant results ($P<0.05$) are discussed.

Characterization of bacterial isolates

Characterization was performed basically with the BIOLOG system and also with the fatty acid profiling.

RESULTS

Ninety-one samples out of 417 showed bacterial growth in the sterility check plating usually containing only a few colonies (1-10), for 11 samples the check showed a more serious contamination which could have interfered with the data obtained for the data on endophytic colonization for that sample. Statistical analysis is presented including all the data since the exclusion of the 11 samples gave very similar results in the analysis.

Endophytic bacterial population levels

Seedling stage

Pooled samples

Mean values of log CFU/g are given in Table 1. Interactions were not found significant. There were significant differences between locations, genotypes and plant parts (root and stem base) (Table 2). The highest endophytic colonization for both root (in four genotypes) and stem (in three genotypes) was observed in the light-clay soil. There were significantly lower CFU/g in the heavy-clay soil than in the sandy and light-clay. Twiggy was the most colonized genotype while JI2202 was the least. Genotypes Twiggy and Fortune significantly differed from JI2202 and Solara. There were no significant differences between Twiggy, Fortune and Norli or JI2202 and Solara or Solara and Norli (Table 2). Counts in roots were significantly higher than in stems.

Individual samples

Mean values of log CFU/g are given in Table 1. Interactions were not found significant. There were significant differences between locations and plant parts (root and stem base) but not for genotypes (Table 2). Also, as for pooled samples, the highest colonization for both root (in four gentoypes) and stem (in three genotypes) was observed in the light-clay location (significantly less CFU/g in heavy-clay than in sandy and light-clay locations). Twiggy was the most colonized genotype and JI2202 the least. Counts in roots were significantly higher than in stems.

Table 1. Mean values of endophytic bacterial populations (log CFU/g fresh tissue) in the root and stem base of five pea genotypes at the seedling stage for pooled (P) and individual (I) samples at three field locations. Analysis of significant differences are given in Table 2

	Fortune		JI2202		Norli		Solara		Twiggy	
	P[a]	I[a]	P	I	P	I	P	I	P	I
Heavy-clay										
Root	5.46[a]	5.02	4.03	3.43	5.20	4.09	5.19	4.96	5.73	5.54
Stem	5.08	4.00	3.48	3.75	5.13	4.17	3.52	3.97	5.39	4.65
Sand										
Root	5.69	5.26	5.84	4.92	5.52	5.93	4.81	5.25	6.38	4.88
Stem	4.78	4.16	4.35	3.57	6.07	5.45	4.33	5.10	6.48	5.63
Light-clay										
Root	5.96	6.13	5.01	5.86	5.80	4.86	5.64	5.59	6.51	6.50
Stem	6.42	5.76	4.83	5.23	5.59	4.43	5.43	5.71	5.89	4.82

[a] log transformation of CFU/g was done for every sample before calculating means

Table 2. Analysis of significant differences in endophytic bacterial populations (log CFU/g fresh weight) between locations, pea genotypes and plant parts at the seedling stage for pooled (P) and individual (I) samples

Location	P	I	Genotype	P	I	Plant part	P	I
Heavy-clay	4.82 *a*	4.33 *a*	JI2202	4.58 *a*	4.54 *a*	Root	5.53 *a*	5.25 *a*
Sand	5.42 *b*	5.03 *b*	Solara	4.87 *ab*	5.15 *a*	Stem	5.10 *b*	4.70 *b*
Light-clay	5.71 *b*	5.56 *b*	Norli	5.48 *bc*	4.82 *a*			
			Fortune	5.55 *c*	5.05 *a*			
			Twiggy	6.09 *c*	5.31 *a*			
Probability	<0.01	<0.001		<0.001			=0.02	<0.001
SED	0.28	0.29		0.33	0.37		0.18	0.14

Means with letters in common do not differ significantly at two-sided test with • =0.05

Flower-pod stage

Pooled samples

Mean values of log CFU/g are shown in Table 3. There were no significant differences between locations. The light-clay location gave the highest counts (Table 4). There was interaction between genotype and plant part. Significant differences were found between genotypes for the root part but not for the stem. JI2202 had the highest colonization in the root and Fortune in the stem. Counts in the middle part of the stem were very similar for all genotypes and they were significantly lower than in the root and the stem base. Values of CFU/g for root and stem base differed significantly only in accessions Norli and JI2202. For half of the treatments there were more CFU/g in the root than in the stem base.

Table 3. Mean values of endophytic bacterial populations (log CFU/g fresh tissue) in the root, stem base and the middle part of the stem for five pea genotypes at the flower stage for pooled (P) and individual (I) samples at three field locations. Analysis of significant differences are shown in Tables 4 and 5

	Fortune		JI2202		Norli		Solara		Twiggy	
	P[a]	I[a]	P	I	P	I	P	I	P	I
Heavy-clay										
Root	5.62	5.89	6.80	5.24	4.10	4.47	5.47	5.77	5.83	7.25
Stem base	5.64	5.25	4.47	4.95	5.35	4.85	5.52	4.74	5.63	5.61
Stem middle	3.45	3.10	3.11	3.46	3.18	2.58	3.11	2.89	3.32	3.41
Sand										
Root	5.56	4.09	6.29	6.41	4.15	4.36	5.60	6.87		
Stem base	5.69	5.90	5.46	6.95	5.41	5.43	5.85	5.96		
Stem middle	4.50	4.02	3.71	4.28	3.18	4.11	4.20	3.81		
Light-clay										
Root	6.80	6.31	6.40	6.07	5.81	5.26	5.48	4.70	5.16	3.98
Stem base	6.23	5.73	5.96	5.62	5.84	5.76	5.29	5.66	5.54	5.06
Stem middle	3.74	2.12	4.15	2.78	4.60	3.92	3.30	2.72	3.47	3.05

[a] log transformation of CFU/g was done for every sample before calculating means

Individual samples

Mean values of log CFU/g are shown in Table 3. There were no significant differences between genotypes, being JI2202 the most colonized (Table 5). There was interaction between location and plant part. There were significantly higher numbers of CFU/g in the root and the stem base than in the middle-stem. Differences between

the root and the stem base were significant only in the sandy location. Six treatments out of 14 gave more counts in the stem base than in the root. The light-clay location gave the highest counts for the root whereas it was the sandy location for the stem.

Table 4. Analysis of significant differences in endophytic bacterial populations (log CFU/g fresh weight) between locations, pea genotypes and plant parts at the flower stage for pooled samples with an interaction genotype x plant part

Location		Genotype	Root	Stem base	Stem middle
Heavy-clay	4.73	Fortune	5.88	5.83	3.81
Sand	4.92	JI2202	6.53	5.31	3.65
Light-clay	5.18	Norli	4.69	5.58	3.70
		Solara	5.53	5.58	3.51
		Twiggy	5.58	5.57	3.44

Average standard error of differences was 0.25 for location. For the interaction genotype x plant part was 0.41, for genotype was 0.34 and for plant part was 0.43

Table 5. Analysis of significant differences in endophytic bacterial populations (log CFU/g fresh weight) between locations, pea genotypes and plant parts at the flower stage for individual samples with an interaction location x plant part

Genotype		Location	Root	Stem base	Stem middle
Fortune	4.72	Heavy-clay	5.49	4.96	3.02
JI2202	5.11	Sand	5.32	6.13	3.99
Norli	4.50	Light-clay	5.60	5.67	2.92
Solara	4.82				
Twiggy					

Average standard error was 0.33 for genotype. For the interaction location x plant part was 0.38, 0.34 for location and 0.39 for plant part

Summary of results on endophytic population levels

The estimated variance components suggested large variation in the data compared with the size of fixed effects (location, plant genotype. and plant part). Variation

between individual plants within a plot was rather small compared with variation between parts within plants (Table 6).

No interactions were detected at the seedling stage (Table 7). The same conclusions for pooled and individual samples were found in respect to differences between locations and plant parts. The light-clay location gave the highest counts and roots were more colonized than stems. Differences between genotypes were significant for pooled samples but not for individual samples. The ranking for means from lower to higher log CFU/g was not equivalent for pooled and individual samples, however in both cases the most colonized genotype was Twiggy and the least was JI2202.

Table 6. Estimated components of variance for fields within a location (σ^2_{field}), plots within a field (σ^2_{plot}), plants within a plot (σ^2_{plant}) and plant parts within a plant (σ^2_{part}) for pooled (P) and individual (I) samples at seedling and flower stages. Standard errors are in parenthesis

	σ^2_{field}	σ^2_{plot}	σ^2_{plant}	σ^2_{part}
Seedling				
P	0.02 (0.08)	0.07 (0.14)		0.54 (0.15)
I	0.00 (-)	0.21 (0.14)	0.08 (0.12)	0.59 (0.11)
Flower				
P	0.00 (-)	0.19 (0.10)	0.06 (0.11)	0.29 (0.06)
I	0.00 (-)	0.12 (0.11)		0.85 (0.13)

When an estimate is negative the component is assumed to be negligible and set to 0

Interactions were detected at the flower stage (Table 7). For pooled samples, there were no significant differences between locations, however, the light-clay location gave the highest counts similarly to the seedling stage. The interaction gentoype x plant part showed differences between genotypes only to be significant for the root and differences between plant parts was only significant for the middle-stem. JI2202 was the most colonized in the root and Fortune in the stem. Differences between genotypes were not significant for individual samples, although JI2202 was the most colonized. The interaction location x plant part showed that the light-clay location gave the highest counts only for the root. For pooled and individual samples, there were significant differences between the middle-stem and the stem base and root; cv. Norli was more colonized in the lower stem than in the root at all locations. There was an increase of population levels at the flower stage (log 5.56 CFU/g) compared to

those at the seedling stage (log 5.14 CFU/g).

Table 7. Differences in mean log CFU/g for endophytic bacterial populations for the factors location, pea genotype and plant part at seedling and flower stages for pooled (P) and individual (I) samples

	Location	Genotype	Plant Part	Interactions
Seedling				
P	SC>EN>GR*	T>F>N>S>J*	RO>SB*	NS
I	SC>EN>GR*	T>S>F>N>J	RO>SB*	NS
Flower				
P	SC>EN>GR	J>F>T>S>N* (RO) F>N,S>T>J (SB) F>N>J>>S>T (SM)	RO, SB>SM*	genotype x plant part
I	SC>GR>EN (RO) EN>SC>GR (SB) EN>GR>SC (SM)	J>S>F>N	RO, SB>SM*	location x plant part

SC, EN, GR:locations with light-clay, sandy and heavy-clay soils respectively; F, J, N, S, T: pea genotypes Fortune, JI2202, Norli, Solara and Twiggy; RO, SB, MS: root, stem base and middle part of the stem; * significant differences, $P<0.05$; NS, not significant

Bacterial types

A total of 570 isolates were used for species identification. BIOLOG was applied to 496 of the isolates and fatty acid (FA) profiling to 177. Of these 177 isolates, 53 did not give any match with BIOLOG, 50 were also identified with BIOLOG with a distance > 0.5 and 74 were only tested with FA. Forty-three of the isolates, for which no match was given by BIOLOG, were characterized by FA with an index > 0.5.

Main groups

Gram-negatives represented 90% of the total bacterial population (Table 8). There was an increase in the Gram-positive population from the seedling to the flower stage: in the sandy location from 4% of the total bacterial population at seedling stage to 8% at flower stage, in the heavy-clay location from 2% to 19%, and in the light-clay location from 3% to 20%. Eighty-two species belonging to 40 different genera were

characterized.

Pseudomonas sp. were predominant (54 % of the total bacterial population) followed by *Pantoea agglomerans* (11%) and *Stenotrophomonas maltophilia* (8%). *Arthrobacter* sp. was the most commonly isolated Gram positive (4.2%) followed by *Curtobacterium* (1.6%).

Pea genotype specificity

Pseudomonas sp. and *Pantoea agglomerans* were detected in all pea genotypes at the three locations (Table 9). *Stenotrophomonas maltopohilia* was isolated from all the genotypes in the heavy-clay location and from four genotypes in the sandy and light-clay locations and *Arthrobacter* sp. from all the genotypes in the heavy-clay location and from three genotypes in the sandy and light-clay locations. Some of the species were only detected in 1-2 genotypes in one location, however, the same species were found in different genotypes at other locations, eg. *Bacillus megaterium* and *Agrobacterium radiobacter* were detected only in the accession JI2202 at the sandy location, while *B. megaterium* was only detected in cv. Norli at the light-clay location and *Agrobacterium radiobacter* only in Solara and Twiggy at the heavy-clay location.

Soil specificity

Acinetobacter sp. were only found in the sandy location (four isolates)(Table 8), *Erwinia carotovora* ssp. *carotovora* (two isolates) in the light-clay location, *Ps. syr.* pv. *phaseolicola* (two isolates) and *Kocuria* sp. in the heavy-clay location (three isolates).

Growth stage specificity

All the 18 *Ps. corrugata* isolates were detected at the seedling stage (Table 8). Three groups were mosly detected at the flower stage: *Arthrobacter* sp. (21 isolates out of 23), *Ps. viridiflava* (13 out of 16) and *Ps. syr. pv. syringae* (11 out of 16). *Agrobacterium* sp. (six isolates) and *Acinetobacter* sp. (four isolates) were only detected at the flower stage.

Table 8. Number of endophytic isolates detected for the locations De Eng (EN, sandy soil), Grebbedijk (GR, heavy-clay) and Schuilenberg (SC, light clay) at seedling (SE) and flower stages (FL)

	EN SE	EN FL	GR SE	GR FL	SC SE	SC FL	Total
Gram-negative							
Acinetobacter baumanii		1					1
Acinetobacter radioresistens		3					3
Agrobacterium radiobacter		2		4			6
Alcaligenes piechaudii	1				3		4
Alcaligenes denitrificans				1		1	2
Alcaligenes xylosoxydans			1	1		1	3
Burkholderia cocovenenans				1			1
Burkholderia galthei					1		1
Buttiauxella agrestis	1		1				2
Cedecea lapagei	1		1				2
Comamonas acidovorans	1						1
Comamonas terrigena	1						1
Enterobacter agglomerans			2				2
Enterobacter amnigenus	1				1		2
Enterobacter cancerogenus					1		1
Enterobacter intermedius	1	1	1		2		5
Enterobacter gergoviae		1					1
Erwinia carotovora ss *carotovora*					2		2
Erwinia rhapontici						1	1
Escherichia vulneris			1				1
Flavimonas oryzihabitans					1	2	3
Flavobacterium johnsoniae					1		1
Flavobacterium indologenes			1				1
Klebsiella oxytoca	1						1
Klebsiella trevisanii		1					1
Janthinobacterium lividum	1						1
Pantoea agglomerans	11	18	6	5	12	8	60
Pantoea ananas		1					1
Pantoea dispersa					1		1
Photobacterium logei				1		1	2
Pseudomonas aurantiaca				2			2
Ps. cichoriii					1		1
Ps. chlororaphis	4	5	1	3	7	1	21
Ps. corrugata	7		9		2		18
Ps. fluorescens	34	25	9	21	16	21	126
Ps. marginalis	4	10		11	27	10	62
Ps. putida	12	3	1	2	4	3	25
Ps. synxantha		1		3	2	2	8
Ps. syringae pv. *helianthi*				1			1
Ps. syringae pv. *lachrymans*			1				1
Ps. syringae pv. *phaseolicola*			1	1			2
Ps. syringae pv. *syringae*		4		3	5	4	16
Ps. tolaasii			3			1	4

	EN SE	EN FL	GR SE	GR FL	SC SE	SC FL	Total
Ps. viridiflava		4	2		1	9	16
Rahnella aquatilis	1		2	1	8	1	13
Rhodococcus luteus				1			1
Serratia fonticola					3		3
Serratia grimessii		1					1
Serratia liquefaciens			3	2		1	6
Serratia plymuthica	1	1					2
Serratia rubidaea	1						1
Sphingobacterium multivorum				1			1
Stenotrophomonas maltophilia		11	6	15	7	4	43
Variovorax paradoxus	1		1				2
Xanthomonas sp.	6				1		7
Xanthomonas campestris pv. *malvacearum*						1	1
Xenorhabdus nematophilus					1		1
Yersinia fredericksenii			1				1
Total Gram-negative	91	93	54	80	109	72	500
Gram-positive							
Arthrobacter agilis				2	1		3
Arthrobacter aurescens		1		2			3
Artrobacter ilicis		1		4		3	8
Arthrobacter oxydans		1		2		1	4
Arthrobacter pacens		2	1	1		1	5
Bacillus sp.	1						1
Bacillus megaterium	1	1				1	3
Bacillus mycoides				1			1
Cellulomonas biazotea		1					1
Cellulomonas fimi					1		1
Cellulomonas flavigena	1						1
Cellulomonas turbata				1			1
Clavibacter michiganense ss *michiganense*						1	1
Curtobacterium albidum						1	1
Curtobacterium flaccumfaciens		1		1	1	5	8
Deinococcus erytromyxa						1	1
Kocuria kristinae				1			1
Kocuria varians				2			2
Microbacterium esteraromaticum						3	3
Microbacterium liquefaciens	1					1	2
Micrococcus lylae				1			1
Paenibacillus polymyxa							1
Total Gram-positive	4	8	1	19	3	18	53
Total G-negative and G –positive	95	101	55	99	112	90	553

Plant part specificity

Ps. syringae pv. *syringae* (11 isolates) was detected only in the stem at the flower stage, *Ps. viridiflava* (10 out of 13 isolates) and *Arthrobacter* sp.(18 out of 21) were mainly confined to the stem at the flower stage (Tables 8 and 9). *Alcaligenes* sp. was detected in the root and the lower stem at the seedling stage (five isolates) but only in the root at the flower stage (four isolates) The ratio between numbers of isolates from the root and numbers of isolates from the stem was *ca* 1 for *Ps. fluorescens* and *Ps. marginalis* for the seedling and the flower stages in the sandy and heavy-clay locations, however, it was *ca* 2 for the same species in the light-clay location. For *Pantoea agglomerans*, the R/S was *ca* 1 for both growth stages and the three locations. For *Stenotrophomonas maltophilia* there were differences depending on the growth stage and location.

Table 9 Bacterial endophytic species at locations De Eng (EN), Grebbedijk (GR) and Schuilenberg (SC) at seedling (SE) and flower stages (FL) from root (RO) and stem (ST) samples in five pea genotypes

Fortune	JI2202	Norli	Solara	Twiggy
EN-SE-RO				
Ent. ammnigenus	*Alc. piechaudii*	*Ced. lapagei*	*Pan. agglom.*	*Pan. agglom.*
Ps. sp.	*B. megaterium*	*Pan. agglom.*	*Ps.* sp.	*Ps.* sp.
Rah. aquatilis	*But. agrestis*	*Ps.* sp.	*Xant.* sp.*	
Xant. sp.*	*Com. terrigena*	*Ser. rubidaea*		
	Pan. agglom.	*Var. paradoxus*		
	Ps. sp.	*Xant.* sp.*		
	Ser. plymuthica			
EN-SE-ST				
Ps. sp.	*Pan. agglom.*	*Ced. lapagei*	*Ent. intermedius*	*Cel. flavigena**
	Bacillus sp.	*Ent. intermedius/*	*Mic. liquefaciens*	*Com. acidov.*
		Kl. oxytoca	*Pan. agglom.*	*Pan. agglom.*
		*Jan. lividum**	*Ps.* sp.	*Ps.* sp.
		Pan. agglom.	*Xant.* sp.*	
		Ps. sp.		
EN-FL-RO				
Ac. radiores.	*Ag. radiobacter*	*Ac. baumanii*	*Kluy.*	
Ps. sp.	*B. megaterium*	*Ac. radiores.*	*cryocrescens*	
	Cel. biazotea	*Pan. agglom.*	*Pan. ananas**	
	Pan. agglom.	*Ps.* sp.	*Ps.* sp.	
	Ps. sp.	*Ste. maltophilia*	*Ste. maltophilia*	

Fortune	JI2202	Norli	Solara	Twiggy
EN-FL-ST				
Art. oxydans	*Ag. radiobacter*	*Ac. radiores.*	*Art. aurescens*	
Art. ilicis	*Art. pascens*	*Cur. flacumf.*	*Pan. agglom.*	
Art. pascens	*Pan. agglom.*	*Kl. trevisanii*	*Ps.* sp.	
Ent. intermedius	*Ps.* sp.	*Pan. agglom.*	.	
Ent. gergoviae	*Ste. maltophilia*	*Ps.* sp.		
Pan. agglom.		*Ste. maltophilia*		
Ps. sp.				
Ser. grimessii				
Ser. plymuthica				
Ste. maltophilia				
GR-SE-RO				
Alc. xylosox.		*Rah. aquatilis*	*Ent. intermedius**	*Ent. agglom.*
E. vulneris		*Ps.* sp.	*Pan. agglom.*	*Pan. agglom.*
Ps. sp.			*Ps.* sp.	*Ps.* sp.
Ser. liquefaciens				*Ste. maltophilia*
GR-SE-ST				
But. agrestis	*Ced. lapagei*	*Art. pacens**	*Pan. agglom.*	*Flav. indologenes*
Pan. agglom.	*Ps.* sp.	*Ent. agglom.*		*Pan. agglom.*
Ps. sp.	*Y. frederiksenii*	*Ps.* sp.		*Ps.* sp.
Ser. liquefaciens		*Var. paradoxus*		*Ste. maltophilia*
Ste. maltophilia				
GR-FL-RO				
Alc. xylosox.	*Bur. cocoven.*	*Ps.* sp.	*Agr. radiobacter**	*Agr. radiobacter*
Art. agilis	*Koc. kristinae*		*Alc. denitrificans*	*Pho. logei*
Cel. turbata	*Pan. agglom.*		*B. mycoides*	*Ps.* sp.
Cur. flacumf.	*Ps.* sp.		*Paen. polymixa*	*Sph. multivorum**
Pan. agglom.			*Pan. agglom.*	*Ste. maltophilia*
Ps. sp.			*Rah. aquatilis*	
Ser. liquefaciens			*Ps.* sp.	
Ste. maltophilia			*Ste. maltophilia*	
GR-FL-ST				
Art. pacens	*Art. ilicis**	*Art. ilicis*	*Agr. radiobacter**	*Art. ilicis*
*Art. aurescens**	*Art. oxydans*	*Koc. varians**	*Art. aurescens*	*Ps.* sp.
Koc. varians	*Mic. lylae**	*Ps.* sp.	*Pan. agglom.*	*Ps. viridiflava*
Ps. sp.	*Ser. liquefaciens*	*Ste. maltophilia*	*Ps.* sp.	*Ste. maltophilia*
Ste. maltophilia	*Ste. maltophilia*		*Rhod. luteus**	
			Ste. maltophilia	

Fortune	JI2202		Solara	Twiggy
SC-SE-RO				
Alc. piechaudii	*Cur. facumf.**	*Ps.*	*Erw. car. car.*	*Bur. glathei**
*Flav. johnsoniae**	*Pan. aggl.*	*Rah. aquatilis*	*Ps.* sp.	*Flav. oryzihab.*
Pan. agglom.	*Ps.* sp.	*Ste. maltophilia*	*Ser. fonticola*	*Pan. agglom.*
Ps. sp.	*Rah. aquatillis*	*Xen. nematop.**		*Ps.* sp.
Ste. maltophilia				*Rah. aquatilis*
				Ste. maltophilia
				Xant. sp.*
SC-SE-ST				
*Cel. fimi**	*Art. agilis*	*Pan. agglom.*	*Alc. piechaudii/*	*Alc. piechaudii*
Pan. agglom.	*Ent.*	*Ps.* sp.	*V. fluvialis*	*Ent.*
Ps. sp.	*cancerogenus**		*Ent. amnigenus*	*intermedius*
Ser. fonticola	*Pan. agglom.*		*Erw. car. car.*	*Ps.* sp.
	Ps. sp.		*Pan. dispersa*	*Ste. maltophilia*
	Rah. aquatilis		*Ps.* sp.	
			Ste. maltophilia	
SC-FL-RO				
Alc. xylosox.	*Alc.*	*Art. ilicis*	*Pan. agglom.*	*Ps.* sp.
Cur. flacumf.	*denitrificans*	*B. megaterium*	*Ps.* sp.	
Cur. albidum	*Pan. agglom.*	*Pan. agglom.*	*Ste. maltophilia*	
Erw. rhapontici	*Ps.* sp.	*Ps.* sp.		
Ps. sp.	*X. c. malvac.*			
Ser. liquefaciens				
SC-FL-ST				
Cur. flacumf.	*Art. oxydans**	*Clav.mich. mich.*	*Cur. flacumf.*	*Artr. pascens**
Cur. pussilum /	*Art. ilicis*	*Flav. oryzihab.*	*Ps.* sp.	*Curt. flacumf.*
Mic. esterarom.	*Dei.*	*Pan. agglom.*		*Pan. agglom.*
Mic. liquef.	*erytromyxa**	*Pho. logei*		*Ps.* sp.
Ps. sp.	*Flav. oryzihab.*	*Ps.* sp.		*Ste. maltophilia*
Rah. aquatilis	*Mic. esterarom.*			
	Pan. agglom.			
	Ps. sp.			

Ps. sp. refers to the presence of some of the following *Pseudomonas* sp.: *aurantiaca, chlororaphis, cichorii, corrugata, fluorescens, marginalis, putida, synxantha, syringae, tolaasii* and *viridiflava*
* Identification by BIOLOG gave a similarity index>0.5 but a distance>5 or identification by fatty acid profiling gave an index<0.5
Not underlined bacterial species were only found in populations<10^5 CFU/g

DISCUSSION

Effect of treatments on total population levels of endophytic colonization

At the seedling stage, the average endophytic colonization level over all treatments for the root and the stem base was log 5.14 CFU/g fresh weight. An increased level was found at the flower stage, with an average of log 5.56 CFU/g fresh weight. These data demonstrate the relatively high and stable endophytic colonization of pea plants during the full growth period of the pea. Reports on endophytic colonization of crop plants show various levels up to log 7 for non-rhizobial populations of red clover roots (Sturtz *et al.*, 1997), up to log 5 for sugar beet (Jacobs *et al.*, 1985), up to log 4.2 for potato tubers (De Boer and Copeman, 1994). McInroy and Kloepper (1991) found a strong increase from seedling stage (log 4 CFU/g) to maturity (log 10 CFU/g) for corn, but for cotton the level at seedling and maturity was log 3, with up to log 6 at an intermediate stage.

The estimates for the standard errors of the fixed effects suggested that there was a large variation in the data. This variation is explicable in terms of the many stochastic factors which affect the opportunity for effective endophytic introduction (Hallmann *et al.*, 1997; Sturtz *et al.*, 2000). Probability of endophytic introduction will only be high during the short period that e.g. a wound formed by a side root can be colonized, while sufficient amount of naturally present rhizosphere bacteria with endophytic potential should be present.

The colonization of the roots of young plants was significantly higher than the stem base (Table 1). Similar observations were reported by McInroy and Kloepper (1995*b*) and Sturtz *et al.* (1997). No significant differences were observed at the flower stage between root and stem base. The middle part of the stem at the flower stage had a significantly lower colonization than the stem base, as was found by Fisher *et al.* (1992). These data, although not conclusive, support the hypothesis that at an early stage bacterial endophytes enter mainly through the root and progressively invade the stem with time, as suggested by Gagné *et al.* (1987). Additional factors which may have interfered are changing conditions in the plant tissue during plant development making the stem base more suitable for colonization through the root system or still unknown conditions favouring biotic or abiotic wounding and introduction at the stem base in a later phase.

Counts derived from five pooled plants were higher, particularly at the seedling stage, than counts derived from plants processed individually. This could be a numerical artefact due to the CFU/g from individual samples being log transformed before calculating means. Furthermore, the five pooled plants were homogenized in 10 ml Ringer's and the individual plants in 5 ml, which may have caused a systematic

error in the extraction efficiency and estimates for CFU/g per fresh weight. The statistical analysis detected significant interactions between the factors studied at the flower stage but not at the seedling stage. The effects of soil type and plant genotype showed clear trends at the seedling stage whereas at the flower stage situation seemed to be more complex due to the interaction of several factors.

Of the five pea genotypes studied, the highest number of endophytes at the seedling stage was found for cv. Twiggy. The colonization level of Twiggy (root and stem base for pooled data) was 1.5 log higher than for JI2202, which showed the lowest colonization. At the flower stage, average colonization of JI2202 for the root, stem base and middle-stem was log 0.35 higher than cv. Twiggy, which was ranked third, however significant differences were only detected for the root. Cultivar effects on total endophytic colonization were reported by Samish *et al.* (1963), Chanway *et al.* (1988) and Adams and Kloepper (1998). However, Gagné *et al.* (1987) found no cultivar effect in analysing the xylem colonization of alfalfa. In an earlier study in which 11 pea cultivars were compared for endophytic colonization, significant differences between cultivars were observed from which cv. Twiggy showed a significant higher colonization level than the other cultivars (not included in this study) at the pod stage (Elvira-Recuenco and van Vuurde, Chapter 5).

A significant soil effect was observed at the seedling stage. The light-clay soil with a humus content of 2.7% gave the highest counts of bacterial endophytes. At the flower stage the same trend was observed but differences were not significant. The effect of soil type on endophyte colonization level has been previously reported by Samish *et al.* (1963) and Mahaffee and Kloepper (1996) who observed a greater colonization by *Pseudomonas* sp. and *Enterobacter arburiae* in sandy soil. In the present study the soil with intermediate texture gave the highest counts. Field crop rotation history (Sturtz *et al.*, 1998, 1999), abiotic conditions such as temperature (Pillay and Nowak, 1997) and/or biotic conditions may have interfered with colonization for the three soil types (Hallmann *et al.*, 1997, Sturtz *et al.*, 2000). Other studies have reported no soil effect (Gagné *et al.*, 1987 and Bell *et al.*, 1995).

Effect of treatments on biodiversity

The complex interaction between soil, plant genotype, growth stage and plant part on the biodiversity of endophytic colonization was analyzed for the major bacterial genera and species. Based on the characterization of 553 isolates, the overall biodiversity in the endophytic populations of pea was high, showing 82 species belonging to 40 different genera. Mundt and Hinkle (1976) found 46 species belonging to 19 genera after characterization of 395 isolates from seed samples of 27

plant species. In our experiments Gram-negative bacteria dominated (average 90% of isolates) with *Pseudomonas* sp., *Pantoea agglomerans* and *Stenotrophomonas maltophilia* as the dominant species. Predominance of *Ps. fluorescens* and *Pantoea agglomerans* was previously reported for pea (Elvira-Recuenco and van Vuurde, Chapter 5). At the genus level *Pseudomonas* sp. accounted to 54% of the total isolates. For the Gram-positives, the incidence at genus level was highest for *Arthrobacter* sp. (40% of the Gram-positive population). All Gram-positive species were present at levels below 2% of the total population. *Bacillus* sp. were only incidentally isolated and formed less than 1% of the characterised isolates. The predominance of Gram-negatives in endophyte populations was also reported for lemon and alfalfa (Gagné *et al.*, 1987). In corn Gram-positives dominated (88%) (Lalande, 1989), but not in the studies of McInroy and Kloepper (1995*a*) where they contributed to 23% of the endophytic community without much variation during the growing season. The high proportion of *Pseudomonas* sp. and very low proportion of *Bacillus* sp in the present study compared to studies by McInroy and Kloepper (1995*a*), Mahaffee (1996) and Mahaffee and Kloepper (1997) might be associated to temperature conditions, with a preference of *Pseudomonas* sp. for cool conditions. Pillay and Nowak (1997) reported that populations of an inoculated endophytic *Pseudomonas* sp. in shoots and roots of tomato were affected by temperature.

For pea there was a change in favour of the Gram-positives for the three soil types with the maturation of the plants. This shift was strongest for the light-clay soil (from 2.7 to 20,0 %) and weakest for the sandy soil (4,2 to 7,9%). Data on endophytic colonization at the species level indicate different ecological behaviour during plant development and population development in the root and the stem base. *Ps. corrugata* was only detected at the seedling stage, populations decreased dramatically with maturation of the crop. *Arthrobacter* sp., *Ps. viridiflava* and *Ps. syringae* pv. *syringae* were mainly detected at the flower stage and were generally confined to the stem, which suggests that they entered the plant through the aerial parts. *Agrobacterium* sp. and *Acinetobacter* sp. were only detected at the flower stage. *Alcaligenes* sp. were found in the root and the stem base at the seedling stage but only in the root at the flower stage, which shows a certain specificity for the root. The number of *Ps. fluorescens* and *Ps. marginalis* isolates in the root and the stem base was very similar for the seedling and flower stage in sandy and heavy-clay soils. However, for the light-clay soil, which gave the highest endophyte population levels, double the number of *Ps. fluorescens* and *Ps. marginalis* isolates were detected from the root than from the stem for both growth stages. *Pantoea agglomerans* showed a similar number of detected isolates for all locations and growth stages, while numbers of *Stenotrophomonas maltophilia* varied according to the field and growth stage.

Data on *Pseudomonas* sp. incidence are available for seeds (Mundt and Hinkle,

1976) and as predominant species in lemon roots (Gardner *et al.*, 1982). In corn *Bacillus* sp. (88%) were more represented in roots than *Pseudomonas* sp. (11%), while *Pseudomonas* sp. were predominant in the rhizosphere (Lalande, 1989). In studies on cucumber (Mahaffee, 1996; Mahaffee and Koepper, 1997) found an incidence of *Pseudomonas* sp. of 16.3% for the rhizosphere soil and of 8.5% endophytically in roots. In this study the genus *Stenotrophomonas* was not reported for the rhizosphere, but incidence in the endorhiza was 6.7%. *Pantoea agglomerans* was the dominant endophyte (60%) in clover foliage (Sturtz *et al.*, 1997). The observations in the present study support the hypothesis that certain types of endophytes like *Ps. fluorescens*, *Ps. marginalis* and *Ps. corrugata* have good opportunities to enter the plant through the root system while others like *Ps. syr.* pv. *syringae*, *Arthrobacter* sp. or *Stenotrophomonas maltophilia* mainly derive from phyllosphere populations but may spread from above-ground parts to the roots.

The increasing interest to apply endophytic bacteria to optimize growth, disease buffering, abiotic stress reduction and/or improved soil fertility has been expressed in several recent reviews (Hallmann *et al.*, 1997, Chanway, 1998, Sturtz *et al.*, 2000; Azevedo *et al.*, 2000; and Kobayashi and Palumbo, 2000). Based on data from these reviews beneficial endophyte effects are described for a variety of endophytic strains such as fluorescent *Pseudomonas* sp., abundantly found in the present study. For beneficial endophyte management in crops, selection of strains should include compatibility with the cultural and environmental conditions. Present data shows significant differences between genotypes of pea and soil types especially at the seedling stage. Compatibility with the plant genotype seems a critical factor (Smith *et al.*, 1998; Sturtz *et al.*, 2000) and promising candidate endophytes should be selected for beneficial effects for a representative panel of the host genotypes in field experiments to obtain robust strains effective in practice.

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Chapter 7

Efficiency of procedures for induction and cultivation of *Pseudomonas syringae* pv. *pisi L-form*

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To be submitted

ABSTRACT

Various procedures were tested for the induction of L-form cells of *Pseudomonas syringae* pv. *pisi*. The first procedure was based on the induction method described for *Pseudomonas syringae* pv. *phaseolicola* L-form (Paton and Innes, 1991). Cell-wall deficient cells were induced in a liquid medium with penicillin. Growth on solid induction medium of these cells developed as granular and vacuolated structures, and characteristic colonies were observed in the first transfer. To overcome the poor growth in subsequent transfers of pv. *pisi* L-form compared to pv. *phaseolicola* and the reversion to the parental type, three new tests were developed: viability of cells was monitored during induction. Optimum induction time in liquid medium with penicillin was much lower for pv. *pisi* than for pv. *phaseolicola*. Viability of L-forms in solid induction medium with penicillin was low and decreased in time; a new inducer, ticarcilin combined with clavulanic acid, which prevented the reversion to the parental type; and a range of concentrations of penicillin and ticarcillin/clavulanic acid by the Spiral Gradient Endpoint for calculation of MICs. Based on the results from these tests an improved induction method for pv. *pisi* L-form is proposed.

INTRODUCTION

L-forms can be described as those bacteria that have either permanently or temporarily lost their ability to synthesize their cell walls and reproduction involves a complicated life cycle associated with marked pleomorphism and filterability (Paton, 1987). They can occur spontaneously only in a few bacterial species (Dienes and Weinberger, 1951; Pachas and Dienes, 1968). The cell wall-less state can be induced for several Gram-positive and Gram-negative species *in vitro* (Madoff, 1981) and *in vivo* (Pachas, 1986) by the action of antibiotics, enzymes, aminoacids and other factors (Strang *et al.*, 1991).

The induction and cultivation of L-forms are difficult due to the osmotic fragility of wall-less cells. Generally, important requirements of the induction medium include the proper consistency of the agar, the presence of animal serum and the correct osmolarity usually provided by sucrose or sodium chloride (Madoff, 1986). However, horse serum inhibited penicillin-induced L-form colony formation in *Staphyloccoccus aureus* when included in an osmotically stabilized culture medium (Shimokawa *et al.*, 1994). High cell densities are also required in the early stages of induction (Neimark, 1986).

L-form cells have a marked different cell morphology (spherical forms and granular growth) from the parental bacteria. Variations may be observed within any one culture which represent a mixture of considerable variability in a still undetermined life cycle (Madoff, 1986). If the inducing agent is omitted from the

medium, the L-forms are likely to revert to normal cell-walled bacteria ('unstable' L-forms). But if after the continued absence of inducing agents they remain in a cell wall-less state, then they are 'stable' L-forms (Paton, 1987).

Grichko and Glick (1999) reported in a review paper that L-forms have been implicated as etiologic agents in both plant and animal diseases and they may also be used to protect plants or animals against disease-causing organisms, or to act as a host bacterium for the production and secretion to the growth medium of recombinant proteins. An important change in L-forms compared to the parental bacteria is often related to pathogenicity. Most pathogenic bacteria, in which pathogenicity is not due to exotoxin production or an extracellular virulence factor, will lose their pathogenicity when totally or partly lose the outer membrane, and generally they regain pathogenicity on reversion to the parental walled form (Neimark, 1986). L-forms have an apparent ability to enter intact plant cells and associate with them (Jones and Paton, 1973; Aloysius and Paton, 1984; Paton, 1987; Paton and Innes, 1991). L-forms of *Ps. syr.* pv. *phaseolicola* unlike the parental type did not elicit a hypersensitive response in tobacco and its association in bean plants (*Phaseolus vulgaris*) conferred induced systemic resistance to halo blight (Amijee *et al.*, 1992).

The purpose of this paper was to optimize procedures for the induction and cultivation of the L-form of *Pseudomonas syringae* pv. *pisi* as a basis for further studies on pea plant and L-form interaction.

MATERIALS AND METHODS

Bacterial strains

Pseudomonas syringae pv. *pisi* race 2, IPO 1631, Plant Research International, Wageningen (ex HRI 202, HRI, Wellesbourne, UK); *Ps. syr.* pv. *pisi* race 6, IPO 1639 (ex HRI 1704B) and *Ps. syr.* pv. *phaseolicola* race 1, IPO 1647 (ex HRI 1281).

Induction and growth media

Composition of liquid and solid media was as in Paton and Innes (1991) using sodium benzylpenicillin at various concentrations. A new inducing method was tested, ticarcillin plus clavulanic acid (Ticarcillin 1500 mg/Clavulanic Acid 100mg, Duchefa).

Terminology

(1) Rods, the cells of wild type parental bacteria with a complete cell wall; (2) Spheres, include spherical bacterial forms resulting from the partial removal of the cell wall (spheroplasts) or total (protoplasts) by an inducing agent in an osmotically protective medium; (3) L-forms, a distinct type of growth derived from bacteria initiated by the complete or partial removal of the cell wall. They resemble the mycoplasma in their colonial morphology, mode of reproduction, growth requirements and ultrastructure (granular, vacuolated) (Madoff and Pachas, 1976).

Induction and growth procedures

Method 1: Induction of L-form with penicillin and viability test

Induction of L-form

Induction of the L-form of *Ps. syr.* pv. *pisi* was made following the protocol described by Paton and Innes (1991) for the induction of the L-form of *Ps. syr.* pv. *phaseolicola*, with some modifications as given in Fig. 1a. Cultures of *Ps. syr.* pv. *pisi* race 2 and 6 and *Ps. syr.* pv. *phaseolicola* race 1 were grown on King's B at 25°C for 24-48 h. Suspensions (*ca* 10^8 cfu/ml), two replicates per treatment, were prepared in (1) LIM+P, liquid induction medium with penicillin (5 x 10^3 units/ml, 3 g l^{-1}), and sterile tap water (STW) as buffer, (2) LIM+P and ¼ strength Ringer's as buffer, (3) STW and (4) Ringer's. STW and Ringer's were used as negative controls. Suspensions were shaken in flasks at 80 rev min^{-1}, 25°C for 16 h to obtain at least 40% spheres and were monitored by interference contrast microscopy.

Ten fold dilutions were made in STW or Ringers up to 10^{-6} and were spiral plated on solid induction medium without penicillin (SIM-P) and on SIM+P (3 g penicillin/l medium) and incubated at 25°C for four days. Then subculturing was done only from SIM-P (there was no growth on SIM+P) to SIM+P with penicillin added at several concentrations (7.5, 6 and 3g l^{-1}). Plates were incubated at 25°C for 5-7 days to obtain pure cultures of L-forms and then subcultured to SIM+P (3g l^{-1}).

Viability test

Due to the limited growth on SIM+P, a viability test was performed for pv. *pisi* strain 1631 to assess viability of spheres, L-forms and rods according to the scheme in Fig.

1a. Suspensions (*ca* 10^8 cfu/ml) were prepared in (1) LIM+P and LIM as buffer, 2) LIM+P and Ringer's as buffer and (3) Ringer's as negative control.

The Bacterial Viability Kit (Live/Dead Baclight L-7007, Molecular Probes) used in this study consists of a mixture of two fluorescent nucleic acid stains, green and red. These stains differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. The green dye labels all bacteria in a population while the red dye penetrates only those bacteria with damaged membranes competing with the green dye for nucleic acid binding sites when both are present. So cells with intact cell membranes will stain green, whereas cells with damaged membranes (cell wall and cytoplasmic membrane) will stain red.

One µl of each component (A and B) of the kit was mixed and added to 200 µl of the shaken bacterial suspension in LIM+P. The total mixture was incubated in the dark for 15 min. A droplet was placed on a glass slide for microscopical examination using incident blue light (Leitz Orthoplan, filterblock I3) and water immersion objective 50x /NA 1.0. Monitoring with the viability kit was done every 2h. Suspensions and 10 fold dilutions up to 10^{-3} were subcultured to SIM-P after 6 h shaking.

Method 2: Optimization of antibiotic L-form induction

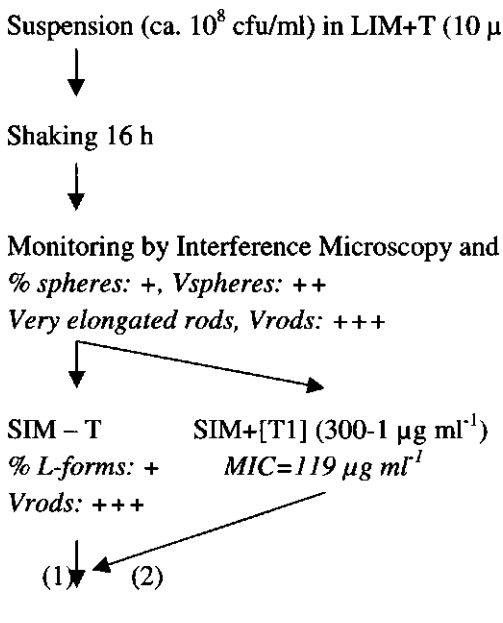
A concentration gradient of penicillin and ticarcillin/clavulanic acid was tested in order to find the optimum concentrations for the induction of the L-form of *Ps. syr.* pv. *pisi*. The steps followed in three protocols are described in Fig. 1b and 1c.

Bacterial suspensions (*ca* 10^8 cfu/ml) were radially streaked from the outer edge of the plate to the centre with a sterile cotton swab on the surface of the SIM containing a radial gradient of antibiotic concentrations, previously deposited by the spiral plater.

Final Inhibitory Concentration (FIC), Nominal Inhibitory Concentration (NIC) and Minimum Inhibitory Concentration (MIC) were determined by the Spiral Gradient Endpoint (SGE) (Weckbach and Staneck, 1987; User guide Spiral System Instruments, 1990). The FIC point represents the last location at which still growth is observed. In some cases there is an outer solid growth on the streak followed by a tail which formed the transition to no growth. The NIC point represents the location at the start of this tail. Standard MIC determination in broth and agar dilution tests is defined as the first dilution that shows no growth. MIC value in spiral plating comparable in magnitude to standard MIC determination can be obtained by multiplying the FIC by a factor of two, using a 10^8 concentration of the test culture.

Plates of 15 cm diameter containing 50 ml medium (layer 3.3 mm thick) were used. The dilution range of the antibiotic was 300 fold. The centre of the spiral was marked as reference point for radial advance (RA) measurements. The various combinations

Fig. 1c. Optimization of L-form induction of *Ps. syr.* pv. *pisi* race 2 (1631) with ticarcillin/clavulanic acid by the determination of MICs (Spiral Gradient Endpoint)



Footnote to Fig. 1a, 1b and 1c

* Modification on method of Paton and Innes (1991)

LIM+P: liquid induction medium with benzylpenicillin, SIM-P: solid induction medium without benzylpenicillin, SIM+P: solid induction medium with benzylpenicillin, LIM+T: liquid induciton medium with ticarcillin/clavulanic acid, SIM-T: solid induciton medium without ticarcillin/clavulanic acid, SIM+[P1] or SIM+[P3]: solid induction medium with a range of concentrations of benzylpenicillin, SIM+[T1] or SIM+[T3]: solid induction medium with a range of concentrations of ticarcillin/clavulanic acid; Growth of culture on agar; '-' indicates no growth; +, ++, +++ scale with increasing growth rate; % spheres, % L-forms: proportion of spheres or L-forms within the bacterial population. (+), 0-5%; +, 5-20%; ++, 20-60%; +++, >60%; Vspheres, Vrods, VL-forms: Viability of cells, only determined for strain 1631. (+), <10%; +, 10-30%; ++, 30-60%; +++, >60%, MIC: Minimum Inhibitory Concentration

Table 1. Concentrations of benzylpenicillin and ticarcillin/ clavulanic acid used for the determination of Minimum Inhibitory Concentrations by the Spiral Gradient Endpoint

Antibiotic	Medium*	Stock Conc.† $\mu g\ ml^{-1}$	Range‡ $\mu g\ ml^{-1}$
Benzylpenicillin	SIM+[P1]	3.6×10^5	1800-6
Benzylpenicillin	SIM+[P2]	3.6×10^5	1800-6
Benzylpenicillin	SIM+[P3]	9×10^5	4500-15
Ticarcillin/clavulanic acid	SIM+[T1]	6×10^4	300-1
Ticarcillin/clavulanic acid	SIM+[T2]	6×10^4	300-1
Ticarcillin/clavulanic acid	SIM+[T3]	2×10^5	1000-3

* Solid induction medium with a range of concentrations of benzylpenicillin or ticarcillin/clavulanic acid (medium codes as used in Table 2 and Fig. 1b and 1c).

†Antibiotic stock concentration=200 x ACmax (highest antibiotic concentration to be tested). The reduction factor is 200 when assuming an agar height of 3.3 at RA= 7 mm, for a C40 spiral plater set at 40 µl

‡ Range of measurenents between RA=7mm and RA=50 mm, from 'ACmax' to 'ACmax/300'

RESULTS

Method 1: Induction of L-form with penicillin (Paton and Innes, 1991) and viability test

Induction of L-form

The results on L-form induction and subculturing are presented in Fig 1a. Shaking of bacterial suspensions in LIM+P (3 g l^{-1}) during 16 h proved to be a good induction procedure to obtain high numbers of spheres (Fig 2A). The intermediate step on SIM-P seemed to be a necessary step between liquid and solid media with penicillin in the growth of the L-form. The percentage of L-forms on SIM–P was less than 20%, although it was higher for undiluted samples. Ringer's showed to be a better buffer than STW both for spheres and rods. A characteristic colony morphology was observed on SIM–P. Very high quantities of inoculum were needed for successful transfer to SIM+P, which made no possible the detection of single colonies on this medium. First colony growth on SIM+P was good, with more than 60% L-forms (Fig. 2B and 2C), but percentage of L-forms and growth on agar decreased in time and with new subcultures. Morphology of L-forms on SIM+P was slightly different from SIM-

P with a more granular and vacuolated structure.

Viability test

Viability staining showed that the optimum exposure time in LIM+P was 6 h in order to find a compromise between a high proportion of spheres and their viability. After 6 h there were *ca* 60% spheres and viability was *ca* 90%. When exposure time increased, proportion of spheres also increased but viability decreased. Viability of spheres was higher when suspensions were prepared in LIM buffer than in Ringer's, while viability of rods was the same for both buffers. Viability of L-form cells in colonies on SIM+P was generally very low and decreased in time (Fig. 2D).

Method 2: Optimization of antibiotic L-form induction

The spiral plating gradient method was used to determine the Final and Nominal Inhibitory Concentrations (Table 2). In the cases in which RA was < 7 mm, FIC and NIC values were approximate since the maximum accuracy of the endpoint analysis is for 7 < RA < 50 mm. MIC values are shown in Fig. 1b and 1c.

Method 2A: Penicillin

This method is presented in Fig. 1b. When subculturing was done from LIM+P to SIM+P, growth on the streak was continuous (without tail). FIC values (Table 2) were much lower compared to those when there was an intermediate step on SIM-P. The FIC for pv. *pisi* (strain 1631) was about four times lower than for pv. *phaseolicola* (strain 1647). These results explain why there was no growth of pv. *pisi* on SIM+P (3 g l^{-1}) in 'method 1' after subculturing from LIM+P since the antibiotic concentration was too high.

When subculturing was done from SIM-P to SIM+P, in the case of pv. *pisi* there was an outer solid growth on the streak followed by a tail which formed the transition to no growth. The range between the NIC and FIC value is very large and might imply a large variance of the resistance frequency distribution of the bacterial population. For pv. *phaseolicola* no tail was detected and the FIC was the same as in pv. *pisi* (Fig. 3). The FIC value '6 g l^{-1}' is on the range recommended by Paton and Innes (1991) for the cultivation of the L-form of pv. *phaseolicola*.

On a new subculturing to SIM+P, FIC values were the same as in the first

subculturing for both pathovars, although a tail was also observed for pv. *phaseolicola* in this case. NIC value for pv. *pisi* was higher in the second subculturing. These results might be due to an increase of resistance of the bacteria to the penicillin (Fig. 3).

Table 2. Nominal Inhibitory Concentration (NIC) and Final Inhibitory Concentration (FIC) of penicillin and ticarcillin/clavulanic acid determined by the Spiral Gradient Endpoint for *Ps. syr.* pv. *pisi* race 2 (1631), *Ps. syr.* pv. *pisi* race 6 (1639) and *Ps. syr.* pv. *phaseolicola* race 1 (1647)

Former medium*	Plate Code†	Bacterial Strains	RA (mm)‡	AC (μg ml^{-1})§
Method 2A				
LIM+P	SIM+[P1]	1631	37.5	FIC‖ = 28.5
LIM+P	SIM+[P1]	1647	27.5	FIC = 109
SIM-P	SIM+[P3]	1631	32	NIC¶ = 59
			5	FIC = 6070
SIM-P	SIM+[P3]	1647	5	FIC = 6070
SIM+P	SIM+[P3]	1631	33	NIC = 129
			5	FIC = 6070
SIM+P	SIM+[P3]	1647	27	NIC=291
			5	FIC = 6070
Method 2B				
LIM+P	SIM+[T1]	1631	18.7	FIC = 59.5
SIM-T	SIM+[T3]	1631	24.2	NIC = 95
			7	FIC=1000
SIM+T	SIM+[T3]	1631	25.2	NIC = 83
			7	FIC =1000
Method 2C				
SIM-P	SIM+P2	1631	29	NIC = 89
			2	FIC = 3924
SIM-T	SIM+T2	1631	14	NIC = 111
			0	FIC > 300

* Medium in which bacterial strains were grown before subculturing to the antibiotic spiral gradient plates. LIM+P, liquid induction medium with penicillin; SIM-P/+P, solid induction medium without/with penicillin; SIM-T/+T, solid induction medium without/with ticarcillin

† Antibiotic spiral gradient plates. Plate code as described in Table 1 and Fig 1b,1c

‡ RA = PR – 13, Radial Advance, radial distance measured from the deposition start circle to location P (location on the plate at which antibiotic concentration in the agar has to be determined), where PR is the distance from the centre of the spiral to location P and 13 mm is the approximate distance from the center of the spiral to the radius where plater deposition starts

§ AC = (SC x DF)/H, average concentration of antibiotic in the agar, where H is the height of agar (*ca* 3.3 mm), DF is the Deposition Factor, a function of RA described on manufacturer's tables and SC is the volume of antibiotic stock solution deposited by the spiral plater at any location on the surface of the plate.

‖ FIC, Final Inhibitory Concentration, last location at which growth is still observed. Concentration at the end of the tail of continuosly growth, excluding the larger separate colonies

¶ NIC, Nominal Inhibitory Concentration, antibiotic agar concentration at the start of the tail. Threshold biocidal concentration

Method 2B: Ticarcillin plus clavulanic acid

This method is presented in Fig. 1c. After 16 h shaking in LIM+T viable spheres were formed and a predominance of very elongated rods of the wild type was observed. These elongated cells indicate an early stage of development of the L-form. It seemed that ticarcillin concentration and/or exposure time in LIM+T were not sufficient for high sphere induction in liquid medium.

However, L-forms could be induced on SIM+T. NIC values did not increase with new subculturing indicating that clavulanic acid is an effective beta-lactamase inhibitor and therefore inhibits the resistance mechanism of the bacterium. FIC values were much higher when there was an intermediate step on SIM-T before subculturing to SIM+T, as for penicillin induction. The viability of L-forms on SIM+T was low as with penicillin.

Method 2C: Direct induction on SIM

A suspension of strain 1631 was prepared in LIM-P, plated and incubated on SIM-P and then subcultured to SIM+P and SIM+T. NIC and FIIC values for both penicillin and ticarcillin were lower than after a previous induction in LIM with antibiotic.

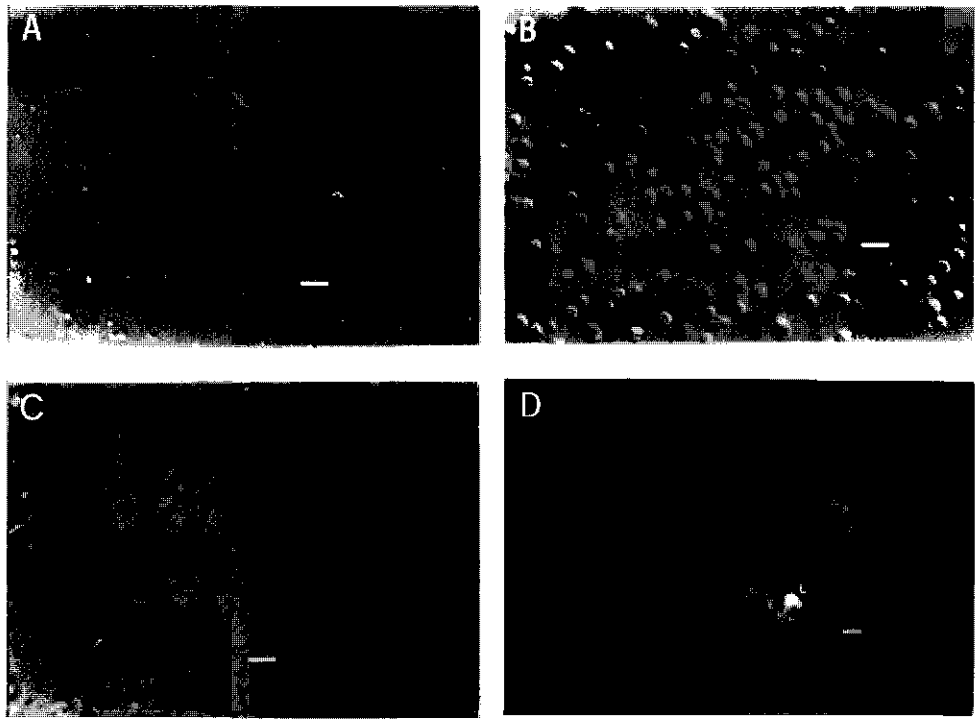


Fig. 2
(A). Cell-wall deficient cells (round) induced in liquid induction medium with benzylpenicillin (5 x 10^3 units/ml) and remaining parental cells (rod, indicated with an arrow) of *Pseudomonas syringae* pv. *pisi* race 2. Bar represents 4 µm
(B). L-form cells induced on solid induction medium with benzylpenicillin (10 x 10^3 units/ml) of *Ps. syr.* pv. *pisi* race 6 after 6 days incubation. Bar represents 4 µm
(C). L-form cells induced on solid induction medium with benzylpenicillin (10 x 10^3 units/ml) of *Ps. syr.* pv. *pisi* race 6 after 8 days incubation. Bar represents 4 µm
(D). Staining of L-form cells with propidium iodide to assess viability. Cells were induced on solid induction medium with benzylpenicillin (10 x 10^3 units/ml) and staining performed after 9 days incubation. In this preparation only one cell (indicated with an arrow) showed to be viable. Bar represents 4 µm

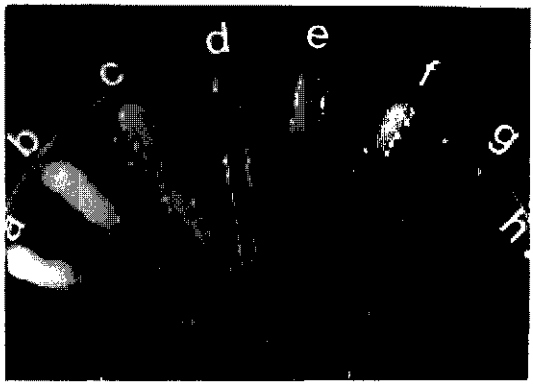
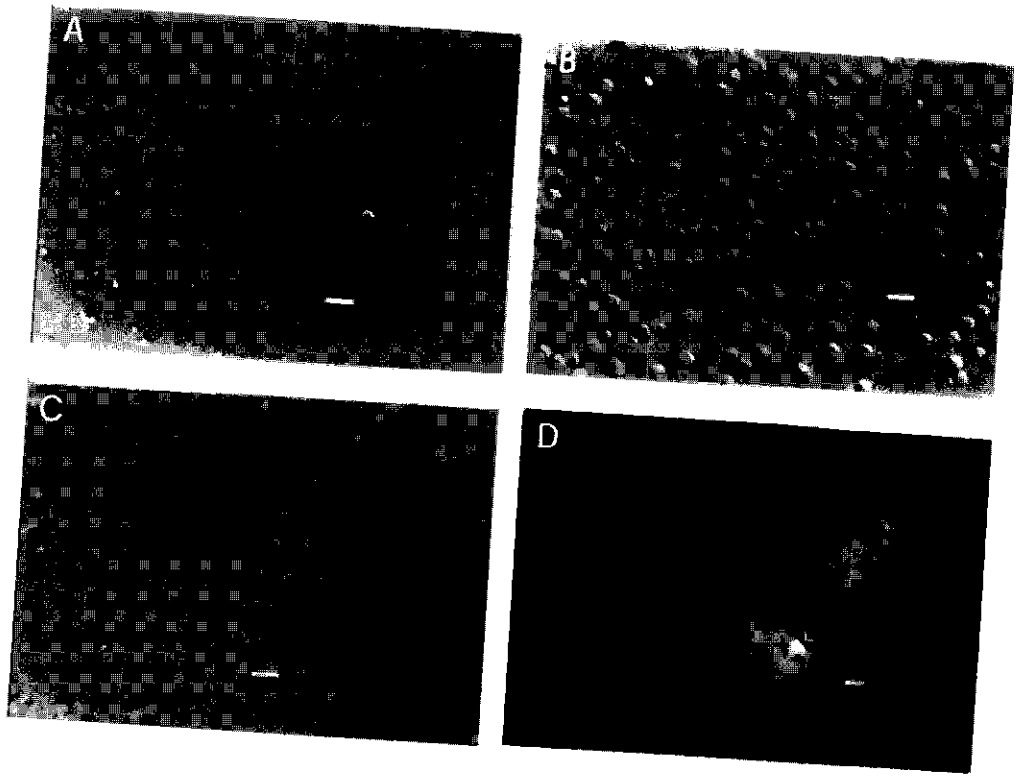


Fig. 3. *Ps. syr.* pv. *pisi* race 2 (streaks a, b, e and f) and *Ps. syr.* pv. *phaseolicola* race 1 (streaks c, d, g and h) on a solid inducition medium with a range of benzylpenicillin concentrations. Streaks a, b, c and d were derived from a solid induction medium with penicillin. Streaks e, f, g and h were derived from a solid induction medium without penicillin. Plate is 15 cm diameter

DISCUSSION

The terminology of the L-forms (L-phase) has been controversial. They have been generally defined as bacteria with modified or no cell walls differing from spheroplasts, protoplasts, and aberrant types. Inherent characteristics are spherical granular and vacuolated bacterial cells which produce colonies on solid media that resemble those of the mycoplasma (Madoff and Pachas, 1976). Colony morphology resulting from surface inoculation on solid media is still the most important identifying characteristic of the L-form (Clasener, 1972). In this study, cell-wall deficient forms (spheroplasts and protoplasts) of *Ps. syr.* pv. *pisi* have been successfully induced in liquid medium with penicillin (5 x 10^3 units/ml) according to Paton and Innes (1991) with some modifications. The optimum exposure time to obtain a maximum number of spheroplasts and protoplasts of pv. *pisi* that are viable was 6 h instead of 16 h used by Paton and Innes (1991) for pv. *phaseolicola.*

First transfer to SIM+P gave a good growth on agar and high proportion of L-



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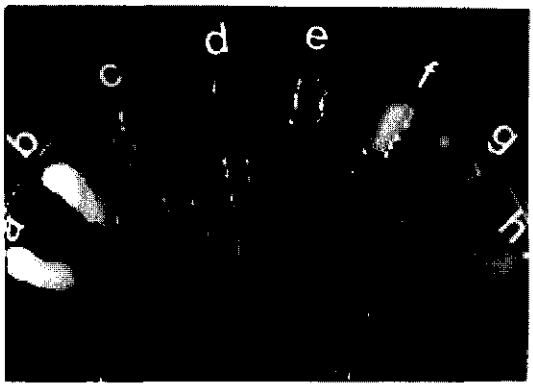

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First transfer to SIM+P gave a good growth on agar and high proportion of L-forms, however, new transfers to SIM+P revealed a poor growth, a decreased number of L-forms, low L-form viability and higher number of bursting cells. Numbers of rods (parental type) increased in these subcultures indicating a possible increase of the

resistance mechanism of the bacterium to penicillin. Landman and Forman (1969) showed that the presence of agar in the medium promoted reversion of *Bacillus subtilis* L-forms and spheres to the rod form, however at a high sphere concentration reversion was inhibited (Landman *et al.*, 1977). In this study high inoculum doses were used for subculturing and the most likely explanation, therefore, is an increase in the resistance mechanism against penicillin.

Due to this, a new inducer, ticarcillin combined with clavulanic acid, was tested. This is an effective combination of a beta-lactam antibiotic (ticarcillin) mainly active against Gram negative like *Pseudomonas spp.* and a beta-lactamase inhibitor of the resistance mechanism of the bacterium (clavulanic acid) which is a high affinity substrate to beta-lactamase. Thus, the resistance of the bacterium by the hydrolysis of the beta-lactam ring of ticarcillin will be avoided.

The sensitivity of pv. *pisi* to penicillin and ticarcillin/clavulanic acid tested for a range of concentrations showed an increase in the resistance mechanism of the bacterium against penicillin. Overall pv. *phaseolicola* could tolerate higher penicillin concentrations than pv. *pisi*. The use of ticarcillin/clavulanic acid proved to be a good inducer to decrease the resistance mechanism since NIC values did not increase in the second transfer but on the contrary slightly decreased. Shmitt-Slomska (1986) reported that L-forms from several bacterial species were resistant to beta-lactam antibiotics. Hubert *et al.* (1971) used gradient plates containing 10 and 30 $\mu g\ ml^{-1}$ of carbenicillin. Because the L-forms tended to revert on these plates, the concentration of antibiotic needed to prevent reversion was 5000 $\mu g\ ml^{-1}$ at the 20^{th} transfer. The use of clavulanic acid allows lower concentration of antibiotic than penicillin for pv. *pisi* and probably less passages are necessary to avoid reversion.

Low viability of L-forms was a limitation in the induction procedure. Strang et al. (1991) reported that a very fine balance existed between the amount of inducer that was bactericidal, that which favoured *Bacillus brevis* L-form induction and that which allowed the bacillary form to grow. The antibiotic inducer balance changed with each subculture and finding this balance for each step seemed to be the key to L-form cultivation. Also some L-forms may require very specific conditions. L-form of *Escherichia coli* specifically requires calcium, and in its absence, cells ceased dividing and eventually lysed (Onoda *et al.*, 2000). Viability of bacterial cells should be monitored during the induction process. Although the viability test used in this study has been successfully used with organisms without a cell wall *(Mycoplasma hominus)*, this is the first report on its use for L-forms.

With the results obtained in this study the recommended induction method for pv. *pisi* is the production of protoplasts/spheroplats in LIM+P (5×10^3 units/ml) (Paton and Innes, 1991) shaking for 6 h at 25°C, 80 rev min^{-1}, transfer to a SIM-P (Paton and Innes, 1991) and after 2-4 days transfer to SIM+T (100-1000 $\mu g\ ml^{-1}$)

(Table 2). Ticarcillin should be used in combination with clavulanic acid in order to switch off the resistance mechanism of the bacterium. Growth improvement on SIM+P subculture can best done using high quantities of inoculum or probably even better by the 'push block' technique used classically for the subculture of mycoplasmas (Fallon and Whittlestone, 1969).

Due to the need to put much research and the lack of further funding, we were not able to carry on with the optimization of the L-form induction protocol for pv. *pisi* to perform the pea-L-form interaction studies. We hope the presented data will facilitate further studies on the induction of L-forms.

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Chapter 8

General Discussion

The aim of the work presented in this thesis was to develop strategies for the control of pea bacterial blight with the emphasis on durability and sustainability. Two approaches were taken for this purpose: (1) development of resistant cultivars based on the combination of race specific and race non-specific resistance and (2) biological control of the pathogen by endophytic bacteria.

To establish guidelines for a resistance breeding programme, it is necessary to be aware of the biology of the pathogen (Chapters 2 and 4), its coevolution with the host (Chapter 2) and the available host genetic resources (Chapters 2 and 3). Race specific resistance has been exploited in pea cultivars with some degree of success, however, at present, the main limitation is the lack of cultivars with resistance to race 6, a race which is becoming increasingly important. The novel resistance found in *Pisum abyssinicum* in a germplasm screening (Schmit *et al.*, 1993), confers resistance to all races of the pathogen, including race 6, and represents the most promising route to disease control. Studies on its mode of inheritance, introgression into a *Pisum sativum* background, and the development of molecular markers to assist in breeding programmes were initiated (Chapter 3).

Biological control of a bacterial disease by endophytic bacteria has scarcely been reported. A necessary step when working with endophytic bacteria is the development of a reliable detection technique (Chapter 5). Whatever the biocontrol mechanism, endophytic bacteria must first be able to colonize the plant at levels greater than 10^4 CFU/g plant fresh weight. Many factors may affect the level of colonization, particularly under field conditions (Chapter 6), and understanding these factors is the basis for an effective biological control programme. Indigenous populations of endophytes isolated at levels higher than 10^4 CFU/g plant fresh weight are potentially effective colonizers and were used for further studies. The use of non-virulent strains of the pathogen may be candidate biocontrol agents. A case studied in the present work is the L-form (cell-wall less variant) of *Ps. syr.* pv. *pisi* (Chapter 7).

DEVELOPMENT OF RESISTANT CULTIVARS TO PEA BACTERIAL BLIGHT

Screening for resistance in Spanish pea germplasm. Frequency of race specific resistance genes

Pea bacterial blight has been known to occur in Spain for some considerable time, and although serious epidemics only occur only every 4-5 years (A. Ramos-Monreal, personal communication), it has caused widespread damage. Despite this, the present study represents the first published record of pea bacterial blight in Spain (Chapter 2).

The incidence of the disease and the frequency of the different races in Spain is still little known compared to France, England or Australia. Races 4 and 6 seem to be the most important races in winter-sown cultivars in Spain, and although this information is based on a limited number of isolations, the situation prompts comparison to Southern France where a similar distribution of races is reported (Chapter 2). However, considerably more isolations need to be made from the different regions of Spain to fully assess the situation.

A study of the resistance characteristics of Spanish *Pisum sativum* germplasm (Chapter 2) was performed on a collection of landraces from the main traditional pea growing areas of Spain. This was done mainly to determine the frequency of known race specific genes but also to identify potential new sources of resistance. The widespread presence of race specific resistance genes and the predominance of R3 in Spanish landraces was similar to that described in a worldwide *Pisum* sp. collection (Taylor *et al.*, 1989). All landraces presented resistance phenotypes that conformed to those previously described in pea cultivars except for landrace ZP-0109, which also showed some partial resistance to race 6, suggesting a possibly new resistance source.

A negative association between the presence of R genes in pea cultivars and *Ps. syr.* pv. *pisi* races has been reported (Schmit, 1991; Hollaway and Bretag, 1995). This is shown by the presence of R3 in all Spanish landraces tested and also its high frequency in commercial cultivars and the corresponding low frequency of race 3. The presence of R4 in 40 % of the landraces would argue against the predominance of race 4 in Spain except that commercial cultivars mostly lack R4.

An increase of race 6 was reported in England over the period 1987-1994, representing 26% of all isolates in 1994 (Reeves *et al.*, 1996). Similarly, race 6 represented 36% of the isolates studied in France (Schmit, 1991), 31%, (Hollaway and Bretag, 1995) in Australia and 50% in Spain (Chapter 2). The lack of cultivars resistant to race 6 and the increasing importance of race 6 is evidence of the association described above and of the changing frequency of specific races due to the use of resistant cultivars carrying race specific resistance genes. The only known established source of resistance to race 6 at present is *Pisum abyssinicum* (Chapter 3) originating mainly from Ethiopia and also from Yemen.

There appear to be no records of the occurrence of pea bacterial blight in Ethiopia, however, it would be interesting to know whether *Ps. syr.* pv. *pisi* is present in Ethiopia, what races and what prompted the evolution of race non-specific resistance in that region. It is expected that during the main growing season of *P. abyssinicum* in the mountains of Ethiopia, weather conditions should be favourable for the disease and potentially therefore disease pressure could be high. In tests for resistance in 14 Ethiopian accessions of *P. sativum* (J.D. Taylor, personal communication), two did not have any resistance genes, seven had R3, six R4 and four R2. Frequencies of R2

and R4 were strikingly similar to those found in Spanish *Pisum sativum* germplasm, however, R3 was found less frequently. Day (1974) and Parlevliet and Zadoks (1977) suggested that during evolution parasites have been kept in check by the need to conserve their hosts to ensure their survival but in modern crops the host-pathogen relationship is quite different from the natural situation. The dramatic change in the host population from genetically very heterogenous to extremely homogenous has completely disturbed the equilibrium between the host and the pathogen.

Race non-specific resistance to pea bacterial blight derived from *Pisum abyssinicum*

Studies on the inheritance of the race non-specific resistance derived from *P. abyssinicum* (Chapter 3) pointed to a model of inheritance which is not polygenic but more likely a major recessive gene together with a number of modifiers. Although segregation at F2 indicated resistance was recessive, probably a single gene, F3 segregation showed a complexity which could not have anticipated at the F2 stage. However, despite this complexity and the difficulty in establishing a clear genetic model, F3 populations segregated in a series of well known ratios indicating the likelihood of a relatively simple underlying genetic control. Vanderplank (1982) suggested that the fact that quantitatively inherited resistance can often respond readily to selection in breeding programmes also implies that the number of genes involved may not always be large.

It is thought that a series of factors might have influenced the complex F3 segregation. Although *P. abyssinicum* has been considered for years as a cultivar group or subspecies of *P. sativum* (Govorov, 1930; Gentry 1971; Westphal, 1972) or an ecotype of *Pisum* (Blixt, 1972), Ellis *et al.* (1998) reported that *P. abyssinicum* is not a subgroup of cultivated *P. sativum*. It is known that there are differences between both genomes in size (Baranyi *et al.*, 1996) and karyotipically (von Rosen, 1944; Saccardo, 1971). Von Rossen (1944) showed that in the main the gene maps agree in both species, however, secondary disturbances considerably increased the difficulty of making a detailed study of the exact parallelism of the gene maps. He also reported on small structural changes in the chromosomes (deletions, small inversions, etc).

At the time the present study was initiated, all the accessions listed as *P. abyssinicum* in the *Pisum* germplasm collection (John Innes Centre, UK) that had been screened for resistance were shown to express race non-specific resistance, these included sixteen accessions originating from Ethiopia (including JI1640), and one from Yemen (JI2202). However, more recently it was noticed that one accession (JI2385) listed as *Pisum* sp., from Yemen, and susceptible to race 6, was in fact *P.*

abyssinicum. It would have been very useful to have included this accession in the crossing programme both in crosses with *P. sativum* and *P. abyssinicum* accessions carrying race non-specific resistance. Crosses between JI2385 and JI2202 in particular would have established the pattern of inheritance of race non-specific resistance in a pure *P. abyssinicum* background, in the absence of possible genomic differences associated with crosses involving *P. sativum*.

Ethiopia is one of the main centres of genetic resources of cultivated plants (Vavilov, 1992). Most cultivated pea types are closely related to wild types found in the Middle East. Secondary centres of diversity have been identified in the highlands of south central Asia and in Ethiopia (Ellis *et al.*, 1998). The Yemenite focus is also included in the Abyssinian center of origin described by Vavilov (1992). This focus reflects influences from both the Ethiopian and the southwestern Asiatic centers (Vavilov, 1992). All the accessions carrying race non-specific resistance originated form Ethiopia, while of the two *P. abyssinicum* accessions derived from Yemen, only one of them carried race non-specific resistance.

In a further attempt to elucidate the genetic control of race non-specific resistance, a molecular analysis of segregant populations was performed using a LTR retrotransposon of pea (PDR1, pea dispersed repeat number 1). Transposons are segments of DNA that move to new chromosomal positions. LTR retrotransposons are closely related to retroviruses, posses a long terminal repeat, use RNA as a transposition intermediate and have been found in fungi, plants, insects, and vertebrates. They are major contributors to the generation of genetic variation (biodiversity) in plants. PDR1 belongs to the *Ty1-copia* group. The degree of sequence divergence in *Ty1* retrotransposon populations between any pair of species is generally proportional to the evolutionary distance between those species (Kumar, 1996). Although the copy number of the PDR1 element is relatively low in pea, there will often be 300 to 400 PDR1 markers segregating in a given cross. PDR1 amplification products are highly polymorphic and provide many markers fairly evenly distributed on all the linkage groups for pea genetics (Ellis *et al.*, 1998). *P. sativum* and *P. abyssinicum* are very distinct when using PDR1 (Ellis *et al.*, 1998). The parental pea lines used in the crossing programme of the present study had not been tested by Ellis *et al.* (1998). Screening of these lines using PDR1 markers confirmed results obtained by Ellis *et al.* (1998), revealing the homogeneity in *P. abyssinicum* when using the PDR1 markers and the divergence from *P. sativum*.

The primary objective of the screening of segregant populations F3, F4 and F5 was to clarify the complex F3 patterns. Although it has not been possible to determine the exact number of genes involved, results suggested that more, but not many more, than one loci were involved in resistance. Six markers were identified, three originating from *P. abyssinicum*, cosegregating with resistance, and three from *P. sativum*

cosegregating with susceptibility. The resistance markers could be used for the selection of race non-specific resistance in marker-assisted breeding The genetic mapping of two of the *P. sativum* markers to linkage group V was possible as they were mapped in two previous recombinant inbred populations (Ellis *et al.*, 1998). Calculations on linkage estimation were made considering resistance as monogenic recessive. It is likely that the major component has been mapped, however both inheritance studies and the presence of markers in the segregants indicated that there are other components (modifiers) involved

Most of the resistance genes cloned and analyzed to date were identified on the basis of their avirulence gene specificity with regard to a single race of a pathogen (Swords *et al.*, 1997). Three genes have proved to be exceptions to this and act in a race non-specific manner: *Bs2* in pepper confers resistance to *Xanthomonas campestris* pv. *vesicatoria* (Swords *et al.*, 1996), *Xa21* in rice to *Xanthomonas oryzae* pv. *oryzae* (Khush *et al.*, 1991) and *mlo* in barley to *Erysiphe graminis* f. sp. *hordei*. The mechanism of race non-specific resistance due to the *mlo* gene is likely to differ from the other two cases. The *mlo* gene is recessive and for its function two recently identified modifier genes (*Ror* genes) are necessary (Freiandelhoven *et al.*, 1996). Described first as an induced mutation (Freisleben and Lein, 1942), it was found later to occur naturally in Ethiopian barleys (Nover, 1968). Inheritance studies with the *mlo* gene have used mutated cultivars as source of race non-specific resistance. Although there are no published reports, crosses between Ethiopian *Hordeum vulgare* and commercial barley cultivars are expected to segregate in a complex pattern (Schulze-Leffert, personal communication). The nature of the race non-specific resistance present in *P. abyssinicum* prompts comparison with *mlo* in barley. In studies of race specific resistance genes isolated from different plant species, there was a high similarity of the related structural domains in the deduced gene products (Dangl, 1995). Accordingly, an interesting approach would be the comparison of the *mlo* gene and the major component of resistance in *P. abyssinicum*.

Expression of resistance to *Ps. syr.* pv. *pisi* in different plant parts

It has been shown that expression of race specific resistance genes R2 and R3 was complete in the stem, while R4 was sometimes expressed incompletely (hypersensitive response associated with a limited water-soaking) (Chapter 4). The incomplete expression of R4 in the stem has been reported previously (Bevan *et al.*, 1995; Hunter, 1996).

Hunter (1996) observed a deviation from Mendelian single gene segregation for stem resistance to races 3 and 4 in some F2 families of crosses cv. Partridge (R3+R4)

x cv. Early Onward (R2); and to races 2 and 4 in F2 families of crosses cv. Vinco (R1+R2+R3+R5) x cv. Hurst's Greenshaft (R4+R6). He suggested that resistance may not be conferred by single dominant alleles but by alleles showing incomplete dominance. This would tend to generate 1:2:1 ratios of phenotypes. The classification of heterozygotes as resistant or susceptible could then vary depending on environmental factors and/or background factors which may or may not be linked to resistance.

Differential expression between stem and leaf/pod resistance was demonstrated for both race specific and race non-specific resistance. Failure to express leaf/pod resistance was most striking in certain R/A combinations, especially R3/A3 and R4/A4 (Chapter 4). Independent genetic control of resistances in stem and leaf/pod seems not to hold: a resistant response in the stem was associated with resistance or susceptibility in the leaf/pod, but a resistant response in the leaf/pod was never associated with a susceptible response in the stem. This suggests that there is a shift on the expression of resistance in leaf/pod which could be associated with modifiers. Mutational analysis has been used to identify genes required for resistance gene function in tomato, *Arabidopsis* and barley (Torp and Jørgensen, 1986; Salmeron *et al.*, 1994; Hammond-Kosack *et al.*, 1994; Freialdenhoven *et al.*, 1994; Century *et al.*, 1995; Parker *et al.*, 1996). Pryor (1987) suggested that resistance genes need to provide two specific functions: pathogen recognition and transduction of the specific signal to the general plant defense pathway. There was no requirement for both functions to be encoded in a single protein

ENDOPHYTIC BACTERIA IN PEA

The printing technique as a routine method for the detection of endophytic bacteria in the pea stem (Chapter 5)

Printing of stem cross-sections gave a semi-quantitative indication of the bacterial population levels and confirmed their endophytic presence. Printing seems to be a good method for semi-quantitative estimation of populations in stems containing 10^4 to 10^7 CFU/g fresh weight. The threshold of the printing technique is 10^3-10^4 CFU/g fresh weight based in comparisons with spiral plating. This represents a constraint to the use of this method when low populations are studied. Also, bacteria are not distributed evenly along the stem but tend to group in certain areas. Therefore populations may be over or underestimated when only a few cross-sections are tested.

This method is recommended for assessment of indigenous populations effectively colonizing the plant and makes possible identification of main types. It is also

recommended for monitoring establishment of introduced endophytes which has been previously marked. It has been used for selecting stems for *in planta* studies on endophytic and pathogenic bacteria with SEM (van Vuurde *et al.*, 1996). Although it is less sensitive than spiral plating, the great advantage of the printing technique is to be a fast method, relatively cheap and suitable for routine application.

Population levels

For both field trials in different years and locations (Chapters 5 and 6), endophytic populations varied from 10^3 to 10^7 CFU/g fresh weight, more commonly 10^4-10^5 CFU/g. There was also large variation between plants of the same cultivar reflecting that colonization is also affected by the biological chance of sufficient cells near a wound or natural opening. There are few reports on the extent and density of colonization by endophytic bacteria, especially for above-ground tissues. Populations in other hosts were also found to be extremely variable (De Boer and Copeman, 1974; Jacobs *et al.*, 1985; Gagné *et al*, 1987; Misaghi and Donndelinger, 1990; McInroy and Kloepper, 1991, 1995*a*; Sturz *et al.*, 1997).

Populations decreased significantly from the stem base to the apex (Chapter 6). Populations in the root were significantly higher than the lower stem at the seedling stage, but not at the flower stage when they were about similar. An increase in endophyte populations was observed at the flower stage compared to the seedling stage (Chapter 6). For roots and stems of sweet corn, McInroy and Kloepper (1995*a*) found an increase from log 4 at seedling until log 10 at plant maturity; whereas for cotton, values of log 3 at seedling, log 5-6 through the season, and a dramatic decrease to log 3 at maturity. Gagné *et al.* (1987) observed no differences in alfalfa during maturation.

Biodiversity

In the first field trial, based on a limited number of isolations from the stem, *Pantoea agglomerans* and *Pseudomonas fluorescens* were the predominant species (Chapter 5). In the second field trial based on a more extensive number of isolates in roots and stems (570 isolates), Gram-negative endophytes represented 90% of the total population (*Pseudomonas* sp., 54%; *Pantoea agglomerans*, 11% and *Stenotrophomonas maltophilia*, 8%). *Arthrobacter* sp. and *Curtobacterium* sp. were the main Gram-positive endophytes and *Bacillus* sp. were scarcely represented. The predominance of Gram-negative endophytes has been often reported: in fruits and

vegetables including peas (Samish *et al.*, 1963), 84% of the population in xylem of lemon-tree roots (Gardner *et al.*, 1982), 94% in alfalfa roots (Gagné *et al.*, 1987), 75% in corn and cotton roots and stems (McInroy and Kloepper, 1995*b*), and 78% in grapevine xylem (Bell *et al.*, 1995). Lalande (1989) observed a different species frequency in corn roots, 88% *Bacillus* sp. and 11% *Pseudomonas* sp. Mahaffee (1996) studied soil, rhizosphere and endorhiza communities for the cucumber crop: in soil *Bacillus* sp. represented 67% of the isolates and *Pseudomonas* sp. 4%; in the rhizosphere, *Bacillus* sp. decreased to 17% with an increase of *Pseudomonas* sp. to 16.3%, while in the endorhiza *Bacillus* sp. were 15.6% and *Pseudomonas* sp. 8.5%.

The main endophyte groups reported are Pseudomoniaceae and Enterobacteriaceae for many crops. Nevertheless, the high proportion of *Pseudomonas* sp. in the present study (54%) compared to Mahaffee studies (8.5%) and McInroy and Kloepper (7%) carried out in Alabama, may have been associated with temperature, with preference of *Pseudomonas* sp. for cool conditions. Population levels of an introduced endophytic *Pseudomonas* strain in shoots and roots of tomato were higher for cool conditions (Pillay and Nowak, 1997)

Three bacterial groups were mainly detected at the flower stage confined to the stem (*Arthrobacter* sp., *Ps. viridiflava* and *Ps. syringae.* pv. syringae). This suggests they entered the plant through the aerial part after the seedling stage. *Ps. corrugata* was only detected at the seedling stage and mainly in the roots (Chapter 6). B. *megaterium* was found in the pea stem (Chapter 5) in one only of the field blocks. In the second field trial (Chapter 6), in a sandy soil, it was found at high populations exclusively in one of the pea lines (JI2202). Hollis (1951) reported *Bacillus megaterium* as an endophyte of potato whereas *B. cereus* was more common in soil and was not found endophytically.

Plant genotype

Significant differences in population levels were observed at the pod stage between cultivars, with cv. Twiggy the most highly colonized (Chapter 5). In a second year field trial at a different location and using different cultivars, except for Twiggy, differences in population levels between cultivars were significant only at the seedling stage. Twiggy was the most highly colonized at the seedling stage and JI2202 at the flower stage, however, JI2202 differed significantly only for the root, and the interaction plant genotype x plant part was significant.

Previous studies on the influence of plant genotype on endophytic colonization are controversial. Gagné *et al.* (1987) and Pillay and Nowak (1997) in alfalfa roots and in tomato roots and shoots respectively did not find any plant genotype effect on

endophytic colonization. Samish *et al.* (1961, 1963) and Adams and Kloepper (1998) found differences between cultivars with several fruits and vegetables and cotton cultivars respectively. Adams and Kloepper (1996) also reported that the occurrence of seed-borne bacterial endophytes within and among cotton genotypes was not necessarily related to cultivar type but was based on physiological and/or morphological components.

Soil type

At the seedling stage, light-clay soil, with a relatively low content of humus (2.7%), gave significantly greater total endophyte counts than sandy and heavy-clay soils. At the flower stage, counts were greater but not significant. Some differences in biodiversity were also observed depending of the soil type (Chapter 6).

Samish *et al.* (1961, 1963) observed that endophytic bacteria may be isolated abundantly in one field and rarely in another, with results varying greatly. When cotton seeds were germinated in agar, populations were higher than in Promix (soil-less potting medium) probably due to the potential effect of microbial competition, moisture and nutrients availability (McInroy and Kloepper, 1995*a*). Addition of chitin resulted in changes in the soil microflora and the endophytic bacterial community (Hallmann *et al.*, 1999). Quadt Hallmann and Kloepper (1996) reported that chemical composition and adsorption capacity of substrate can affect bacterial fitness and motility. Mahaffee and Kloepper (1996) observed that colonization of *Ps. fluorescens* and *Enterobacter arburiae* was greater in sandy soils. *Bacillus subtilis* subsp *globigii* in cabbage roots, could be recovered for up to 28 days only when grown in vermiculite, while in the field they were recovered the whole growing season (Wulff, 2000). Studies by Gagné *et al.* (1987) and Bell *et al.* (1995) reported no substrate effect.

Isolation Media

For the studies in the first field trial (Chapter 5), three isolation media were tested which supported the growth of particular bacterial types: 5% TSA (for heterotrophic types), R2A (for oligotrophic) and SC (for fastidious organisms). The medium 5% TSA gave a significantly 17% higher plating efficiency than R2A and SC, equivalent to an increase of 0.07 in log counts. McInroy and Kloepper (1991) found no differences between full TSA, R2A and SC, but subsequently McInroy and Kloepper (1995*a*) described significantly greater populations on R2A and SC than on full TSA.

Sturz *et al.* (1997) reported lower populations on R2A than full TSA. The medium 5% TSA was used by Adams and Kloepper (1996) for isolation of cotton endophytes. In the studies during the second field trial 5% TSBA was used, as it seems to support the growth of wide range of endophytic bacterial species in pea.

Assays on disease reduction

Preliminary experiments on disease buffering by introduced endophytes (not reported in this thesis), were conducted for six isolates derived from the first year field trial (Chapter 5); seven biocontrol top isolates of the collection at Department of Phytopathology, Auburn University, Alabama (courtesy Prof. J. W. Kloepper) and several combinations of these 13 isolates.

These isolates were tested for antagonism *in vitro* by spot inoculation on King's B and TSBA media pour plated with 1% *Ps. syr.* pv. *pisi* race 2 suspension (*ca* 10^9 CFU/ml). They were also tested under glasshouse conditions together with the chemical inducers: Actigard, BABA (DL-B amino-n-butyric acid) and salycilic acid at several concentrations. Several inoculation techniques for both the biocontrol agent and the pathogen were tested. For the endophytes, seed inoculation with vacuum infiltration, priming with 2% methylcellulose and 0.1% Silwet plus endophytic suspension (ca 10^8 CFU/ml), leaf inoculation and a soil drench were tested. Pathogen challenge was usually made at the 4^{th} node stage. Concentration of the pathogen suspension was critical and it was necessary to find an optimal challenge with sufficient concentration for the development of symptoms in the glasshouse assay but which was not so aggressive that the biocontrol agent was unable to exert a noticeable positive effect. In order to get symptoms consistently through wounding plus leaf spraying, it is necessary to use a concentration of at least 10^7 CFU/ml.

In a series of three experiments disease reduction was consistently observed for Actigard (0.40 mg/ml), however, there was also a reduced fresh weight at the seedling stage. A combination of isolate S321 (*Ps. fluorescens)* and isolate S355 (*Bacillus pumilus)* gave the best performance with the highest disease reduction (a disease index of 0.38 compared to 1.2 of the disease control). Isolate S355 also showed the highest antagonism *in vitro* of the 13 isolates tested individually.

TOWARDS A DURABLE AND SUSTAINABLE CONTROL OF PEA BACTERIAL BLIGHT

Durable genetic resistance

Durability of resistance is a primary concern for breeders. Durable resistance can be defined simply as resistance that lasts a long time. Absolute proof of durable resistance is therefore not possible. According to Johnson (1979), recognition of durable resistance is a retrospective judgement and can only be recognized in those lines that have been widely grown for a reasonably long period. Parlevliet and Zadoks (1977) stated that differences in durance of resistance result from the inability of the pathogen population to have the required gene or genes, or from the inability to exploit the gene or genes that are present in low frequencies. Stability of resistance is assumed to be highest when many resistance genes and pathogenicity genes are involved, and when recombination in the pathogen is restricted. However, durable resistance can also be under simple genetic control. Stability results from the absence of a gene-for-gene relationship, so that changes in a number of parasite genes would be required to overcome the effects of a single host resistance gene (Crute, 1985)

Race specific resistance controlled by single genes is readily handled in breeding programmes and is generally expressed as a hypersensitive reaction. A serious disadvantage is the sudden appearance of new races which, either following mutation, or more likely from existing small virulent populations increase rapidly in response to the selection exerted by genes for high resistance (Wood, 1982). Nevertheless, genes for race specific resistance are a valuable natural resource if they are used within a diverse background and, if possible, are used in combination with race non-specific resistance (Robinson, 1971). The two different types of disease resistance found in natural populations (race specific and race non-specific resistance) are not inevitable alternatives (Burdon, 1996). Clifford (1975) stated:'in common with other workers, the author accepts the convenience of cataloguing resistance in two types. Nature, I am sure, never intended this division'.

Hayes (1973) indicated that 'the incorporation of the best available genes for race specific resistance into genotypes having the highest possible level of race non-specific resistance provides the best long term solution especially in the light of the indication that major genes themselves may exhibit a 'ghost' effect when present in backgrounds with moderate to high levels of non-specific resistance'.

Race specific resistance to pea bacterial blight has been defined on the basis of a gene-for-gene relationship. However, the test for resistance was based on plant responses to stem inoculation. The differential responses in other plant parts (Chapter 4) needs more investigation, especially on genetic control, nevertheless findings

obtained in this study provide much relevant information which needs to be considered when establishing a breeding programme. Resistance with a consistent performance in the leaf/pod to races 3, 4 and 6 can not be achieved with single race specific resistance genes or race non-specific resistance, however, the combination of race specific genes R3+R4 with their additive effect provides leaf/pod resistance to races 3 and 4. Race 6 is not as aggressive in the leaf as race 4, and the susceptibility of *P. abyssinicum* to race 6 under field conditions is relatively low (Chapter 4). Resistance in the stem to all races is provided by race non-specific resistance, which can be reinforced by the combination of race specific resistance genes. Race 4 is the most aggressive race and both race specific and non-specific resistances are 'leaky' on some instances, however, it is expected that the combination of both types of resistance will reduce this partial susceptibility. It has been shown that race specific genes (Chapter 3) exert a 'ghost effect' on resistance to race 6. Screening for race specific genes R2, R3 and R4 can generally be performed by pathogenicity tests and reactions easily scored. Screening for race non-specific resistance is more difficult and the use of molecular markers is recommended in selecting for this trait.

For the Spanish situation, the pea cultivars used on relatively large farms are mainly imported and many of them are not well adapted to Spanish agronomic conditions. The research group at Servicio de Extenxión Agraria, Valladolid, have worked on the development of new cultivars ('Cea' and 'Esla') better adapted to Spanish growing conditions. However, both are susceptible to races 4 and 6 of bacterial blight. Progenies of crosses between these cultivars and resistant F5s carrying race non-specific resistance and race specific resistance genes are now available (Chapter 3).

Biocontrol by endophytic bacteria

Factors such as soil or plant genotype may affect colonization by endophytic bacteria (Chapters 5 and 6). Changes in these factors will potentially influence the performance of the introduced endophyte. Recent studies show an increasing interest for the relationship between the plant genotype and the efficiency of colonization by beneficial bacteria. Sturz *et al.* (2000) suggested that selecting for yield characteristics and disease resistance is likely to have a collateral effect on selection for host-endophyte interaction. Siciliano and Germida (1999) reported that the composition of the root bacterial community of canola differed between transgenic and non-transgenic cultivars, and this cultivar effect was more pronounced in the root interior compared to the rhizosphere community. A significant variation was found among a recombinant inbred population of tomato on supression of *Pythium torulosum* by

Bacillus cereus and also a significant variation for resistance to *P. torulosum* (Smith *et al.*, 1999). Cultivar-specific of the growth promoting effect by endophytic bacteria has been described (Bensalim *et al.*, 1998). It has been shown in the present study that pea genotype may affect significantly endophytic populations.

Although endophytes are less exposed to fluctuations in environmental conditions than epiphytes, changes in the microflora of soil, root and aerial epiphytic populations may also affect endophytic colonization. Interactions between internal and external microfloral populations have been described (Sturz and Christie, 1995; Shishido *et al.*, 1995). Pillay and Nowak (1997) reported a negative effect of high temperature on the population of an endophytic *Pseudomonas* strain in tomato roots. Therefore, it is important to use as biocontrol agents those microorganisms indigenous to the soils and plants of the region. A selection of indigenous endophytes strains originating from the pea field trials (Chapter 5 and 6) and shown to be effective colonizers should be introduced in the plant and screened for biocontrol of *Ps. syr.* pv. *pisi* under field conditions. Candidates are isolates S319, S321 and S355 which showed disease reduction *in planta* under glasshouse conditions

The potential use for biocontrol of avirulent strains of the pathogen was intended to be studied with the L-form of *Ps. syr.* pv. *pisi*. This type of research is still in a preliminary phase and in this thesis only an improved procedure for the induction of L-form cells *in vitro* is reported (Chapter 7). This was shown to be a complex process and because of their fragility and the possibility of reversion, practical applications in commercial pea cultivation are still very speculative.

Bacteria from the phylloplane have provided some biological control (Andrews, 1990; Wilson and Lindow, 1993), however, the majority of bacterial biocontrol agents have been selected from among the rhizobacteria (Beauchamp, 1993). Most of these epiphytic biocontrol agents have not fulfilled their initial promise due to poor rizosphere competence and instability of these agents in long-term culture (Sturz *et al.*, 2000). Internal plant tissues are a more protected environment than plant surfaces where exposure to extreme environmental conditions and microbial competition are major factors limiting long-term bacterial survival. Bacterial endophytes could partially overcome these constrains for a consistent biocontrol performance.

Future research: Potential integration of genetic resistance and biocontrol

The information and materials developed in this genetic study should enable the deployment of a broad based and likely durable resistance to bacterial blight into pea breeding programmes using current technologies. In particular, this resistance should permit the growing of winter peas in regions where the disease is at present a major

constraint. With the likely future development of transformation in peas it should be possible to transfer numbers of genes into a range of pea cultivars without the need for time consuming backcross programmes. This use of GM technology should be acceptable since only indigenous *Pisum* genes would be involved.

This study has resulted also in an understanding of the ecology of endophytic bacteria in pea and the selection of indigenous effective colonizers. It has shown that it is necessary to be aware of factors such as soil type and plant genotype when aiming for a biocontrol programme with a consistent practical application under field conditions.

Integration of genetic resistance (race specific and race non-especific) with endophytic bacteria are complementary measures for the control of pea bacterial blight. Furthermore, it is expected that biocontrol will enhance durability of the genetic resistance by the reduction of the pathogen population in a race non-specific manner.

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Summary

Pea bacterial blight (*Pseudomonas syringae* pv. *pisi*) occurs worldwide, mainly under cool and wet conditions. Although considered of moderate importance, the whole crop can be lost when damage is caused at the seedling stage, particularly in winter sown crops. Disease control measures used include disease avoidance through seed testing and the deployment of resistant cultivars with race specific resistance genes. The frequency of occurrence of specific races of *Ps. syr.* pv. *pisi* varies geographically and there seems to be a negative correlation with the frequency of matching race specific resistance genes. An increasing incidence of race 6 has been reported, for which at present there are no resistant cultivars available. Development of alternative methods of control and integration of control measures are necessary for a more effective and durable control of this disease. In the present study two major approaches have been investigated: (1) a genetic approach, through the study of race non-specific resistance present in *Pisum abyssinicum*, which confers stem resistance to all races of the pathogen, its mode of inheritance and introgression into commercial cultivars of *P. sativum* in combination with race specific resistance; (2) a biological control approach, through the study of the ecology of bacterial endophytes from pea and their potential use as biocontrol agents of pea bacterial blight.

GENETIC RESISTANCE

Resistance to pea bacterial blight in Spanish *Pisum sativum* germplasm

Resistance to pea bacterial blight in Spanish *Pisum sativum* germplasm was screened to study frequency of race specific resistance (R) genes and identify potential new sources of resistance. Screening was done by inoculation with seven *Ps. syr.* pv. *pisi* races onto ten landraces originating from different geographical areas of Spain and considered to be representative of the traditional pea crop in Spain. Resistance phenotypes exhibited by the different landraces mainly conformed to those previously described in pea cultivars and corresponded to the established genotypes R3, R2+R3, R3+R4, R2+R3+R4. The exception was the landrace ZP–0109, a genetic mixture with some partial resistance to race 6, with some evidence of the presence of the poorly documented R genes, R5 and/or R6. The most common resistance gene was R3, present in all the landraces. R4 was present in four and R2 in three of the landraces tested. None was completely susceptible or resistant to all races.

In addition, genetic diversity for resistance to *Ps. syr.* pv. *pisi* was compared with diversity for five morphological traits selected as being largely independent of environmental influences: flower colour, primary seed colour, shape of seed, degree of pod curvature and shape of pod tip. Variation for resistance within landraces was

generally low (less than 10% for seven of the landraces). The same landraces that showed higher variation for resistance also showed variation for morphological traits. Landrace ZP-0109 appeared to be highly diverse for all the characters studied. This study also represents the first published record of the presence of *Ps. syr.* pv. *pisi* in Spain from a collection of 16 isolates originating from the Northwest of Spain (Valladolid), eight being identified as race 6 and eight as race 4.

Race non-specific resistance to pea bacterial blight derived from *Pisum abyssinicum*

Inheritance of potential race non-specific resistance (RNSR) to pea bacterial blight derived from *Pisum abyssinicum* was studied through a crossing programme between two *P. sativum* cultivars ('Kelvedon Wonder' and 'Fortune') and two *P. abyssinicum* accessions (JI2202 and JI1640). 'K. Wonder' is susceptible to all races of *Ps. syr.* pv. *pisi,* 'Fortune' is resistant to all races except race 6 (with race specific genes R2, R3 and R4), and JI2202 and JI1640 are resistant/partially resistant to all races, including race 6. Screening for resistance of progenies was done by stem inoculation with race 6.

The F1 generation was generally susceptible as expected. There were reciprocal differences in F1 plant fecundity, which was lower for crosses involving *P. abyssinicum* as the female parent. Segregation ratios in F2s derived from crosses with 'K. Wonder' were predominantly 1:3 Resistant:Susceptible (R:S), ($P>0.05$). The segregation ratio for the morphological marker plant height was as expected, 3:1 Tall:Dwarf (T:D), ($P>0.5$) The exception was the cross JI2202 x KW, which did not fit the 1:3 R:S, nor the 3:1 T:D and also had a lower F1 fertility. F2s derived from crosses with 'Fortune' behaved somewhat differently, with a higher number of resistant plants than in crosses with 'K. Wonder'.

Segregation patterns at F3 did not conform to the expected segregations for a single recessive gene. F3 plants produced from selected resistant and susceptible F2s segregated in a complex pattern conforming to a series of ratios: three segregating categories (13:3, 1:1, 3:13 R:S) for F3s derived from resistant F2s and two segregating categories (1:1, 3:13 R:S) or uniformly susceptible for F3s produced from susceptible F2s. For F3s derived from unselected F2s, the same segregating categories were observed, except that in this case two families were found to be uniformly resistant, however, the relatively low numbers of plants could not preclude a hidden 13:3. From both sets of data the three observed segregation ratios were present in progenies derived from the same F1 plants.

F5 families were produced from resistant selections at F2 and F3 based on the

preponderance of resistant plants in F3 families, however, at the time these selections were made it was not yet recognized that these families conformed to a F3 segregation ratio of 13:3 R:S. Twenty-seven out of thirty families were uniformly resistant. Selected F5 resistant plants, based on strong resistant responses and no water-soaking, were crossed with the parental lines, with commercial cultivars: Solara (with race specific resistance gene R3), Belinda (R3), Cea (R3), Esla (R3), Frisson (R2 and R3), 'Fortune' (R2, R3 and R4), Ballet and Orb (unknown resistance), and with the accession JI15 (R1).

The presence in 'Fortune' of race specific resistance genes which confer resistance to all races of *Ps. syr.* pv. *pisi* except race 6 appeared to enhance resistance to race 6 in crosses with *P. abyssinicum.* This could be observed as an excess of resistant plants at F2 compared with crosses involving 'K. Wonder'. The only two families that were apparently uniformly resistant at F3 were both derived from the crosses with 'Fortune'.

An apparent linkage of dwarfing habit with resistance was observed. The number of plants that were dwarf and resistant at F2 was higher than would be expected for independent assortment. However, for only one cross (JI2202 x FORT) did a significant probability value indicate linkage. For F3s produced from selected F2s, all the plants belonging to F3 families of the 13:3 R:S category were either uniformly dwarf or segregating for height but not uniformly tall. At F5, 22 out of 27 of the uniformly resistant families were uniformly dwarf.

Due to the complexity of the segregation patterns, a molecular approach was taken to elucidate possible genetic control. This was done using a *Ty1-copia* group retrotransposon of pea (pea dispersed repeat number 1, PDR1) and SSAP (sequence specific amplification polymorphism) markers. Amplification products of *P. sativum* parental lines were shown to be similar to those obtained from a well characterized *P. sativum* cultivar (JI399) while the two *P. abyssinicum* lines were identical to previously characterized *P. abyssinicum* accessions. Screening for markers in segregant F3, F4, and F5 populations was aimed at single plants that were homozygous either for resistance or susceptibility.

Three markers cosegregating with resistance and three with susceptibility were identified. All the plants tested in which the three *abyssinicum* markers were present and the *sativum* markers were absent were resistant. These plants mainly derived from resistant selections from the 13:3 R:S F3 category. However, half of the plants in which some of the *P. sativum* markers were present, were derived from the 1:1 or 3:13 R:S categories. Linkage intensity was estimated considering a single group of markers (resistance as monogenic recessive) and recombination frequencies were estimated treating F2 and RI models as upper and lower bounds. This analysis suggested that these six markers lie within *ca* 25 cM of the locus which is the major determinant of

resistance; however, it is possible that some of the phenotypic scores were influenced by modifier loci. Two of the *P. sativum* markers were mapped using two recombinant inbred populations (JI15 x JI399 and JI281 x JI399), locating the susceptible allele to linkage group V in *P. sativum.* The distance between these two markers was difficult to estimate due to the different recombination frequency from the two crosses but they are probably 20 cM apart. The alleles *r* (wrinkled seed), *pa* (foliage colour), or *det* (determinate growth) could be used as classical markers for the introgression of the resistance trait.

According to the results described above the proposed model of inheritance is based on a major recessive gene present in *Pisum abyssinicum* and a set of modifers, most likely two, which could be present in *P. abyssinicum* and/or *P. sativum.*

Responses to pea bacterial blight in different plant parts: glasshouse and field studies

Resistance to pea bacterial blight has usually been screened using a stem inoculation technique on the assumption that responses would be the same in other plant parts. However, observations of stem inoculations very close to the junction of the stipule with race 3 on a cultivar carrying R3, sometimes showed a water-soaked (susceptible) response spreading into the stipule while the stem showed the expected necrotic (resistant) response. More extensive studies were therefore undertaken to determine the extent of the differential responses to the seven races of pea bacterial blight in stems, leaves and pods. Experiments were performed under glasshouse conditions using a range of pea accessions (*P. sativum* and *P. abyssinicum*) carrying either single race specific genes or varying combinations of race specific genes or race non-specific resistance.

It was shown that a resistant response in the stem was not always associated with resistance in leaf and/or pod. This was observed for both the race specific and non-specific cases. Race specific resistance genes conferred resistance in the stem in a consistent manner, however, there was a variability in leaf and pod responses which depended of the matching R-gene and A-gene (avirulence gene in the pathogen) combination. Race specific resistance gene R2 generally conferred resistance in all plant parts to races 2, 5 and 7 (each of which carry A2). Resistance genes R3 and R4 individually did not, in most cases, confer complete leaf and pod resistance, however, R3 in combination with R4 had an additive effect in the resistance. Accessions carrying race non-specific resistance were resistant to races 2, 5, and 7 in all plant parts and resistant in the stem but with variable resistance/susceptibility in leaf and pod to races 1, 3, 4, and 6. This suggests that race non-specific resistance does not

always express in leaf and pod and that JI2202 and JI1640 might carry also R2.

In addition, the effects of environmental conditions on resistance expression were studied in the field in the autumn/winter of 1998-99. The experiment was made with pot grown plants of 'K. Wonder', JI2202 and JI1640 inoculated with the three most important races: 2, 4 and 6. Inoculation was done by spraying the bacterial suspensions but without previous wounding in an attempt to simulate natural infection. Eight weeks after inoculation, all 'K. Wonder' plants were dead while *P. abyssinicum* plants were alive and in generally good condition, although plants inoculated with race 4 showed some stem snapping. Frost damage was more severe in 'K. Wonder' than in *P. abyssinicum* and since frost damage is reported as a factor that favours infection, frost tolerance in *P. abyssinicum* constitutes a complementary factor for disease reduction in winter sown crops. Race 4 was shown to be the most aggressive race both in the glasshouse and under field conditions. The leaf susceptibility of *P. abyssinicum* to race 6 was much less obvious under field conditions. This suggests that the combination of race non-specific resistance from *P. abyssinicum* with the race specific genes R3+R4 could provide a very potent combination even under autumn/winter conditions.

POTENTIAL FOR BIOCONTROL BASED ON ECOLOGY OF ENDOPHYTIC BACTERIA

Detection of indigenous endophytic bacteria in pea cultivars

The natural incidence of endophytic bacteria in pea stems and procedures for isolation were studied in a field variety trial in the south of the Netherlands. A fast screening technique for the detection of indigenous endophytic bacteria in pea stems was developed involving printing on 5% TSA (Trypticase Soy Agar) of stem cross-sections that were previously surface disinfested with 1% available chlorine plus 0.1% Tween 80 for 5 min. Eleven cultivars at the flower stage were screened from the stem base to the apex. The printing of cross-sections gave a semi-quantitative indication of the population levels. For five cultivars endophytic colonization was detected, with cv. Twiggy showing the highest and most consistent colonization, differing significantly from other cultivars. Cv. Twiggy was examined further by testing twenty plants at the pod stage using the printing technique from the stem base to the apex. In addition, one section from the third and the fourth internode was surface disinfested, homogenized and spiral plated on three media (5% TSA for herotrophic bacteria, R2A for oligotrophic and SC for fastidious). Endophytic populations decreased acropetally. Populations varied in the range 10^3 to 10^7 CFU/g fresh stem tissue, being usually from

10^4 to 10^5 CFU/g. Populations in one of the field blocks differed significantly from the other blocks. Over a series of 30 stem extracts, recovering efficiency was 17% significant higher for 5% TSA than for R2A and SC. The main species were *Pantoea agglomerans* and *Pseudomonas fluorescens. Ps. viridiflava* was mainly detected in one field block and *Bacillus megaterium* exclusively in this block. The efficiency and reliability of the printing technique were compared with the traditional plating method. The relationship between counts in prints and CFU/g by spiral plating showed that printing on 5% TSA is a good method for semi-quantitative estimation of populations in pea stems containing 10^4 to 10^7 CFU/g fresh stem tissue.

Factors influencing endophytic bacterial populations in pea

Plant genotype apparently plays a role on the indigenous endophytic bacterial populations in pea. Further studies on the effects of cultivar type was combined with studies on endophytic colonization related to soil type, growth stage of the crop and plant part at three field sites in the central-east of the Netherlands with different soil characteristics (texture, organic matter content and pH). The pea lines were four *P. sativum* cultivars with a variety of commercial purposes: Solara, Norli, 'Fortune' (parental line in the crossing programme), and Twiggy (the cultivar with highest endophytic colonization); and one *P. abyssinicum* accession, JI2202 (parental line in the crossing programme). At each site, there were two fields with two blocks per field and five plots per block, corresponding to a randomized complete block design. Plants were sampled at seedling and flower stage. Root and stem parts were processed pooled (from 5 plants) or individually by surface disinfestation and spiral plating on 5% TSBA. Characterization of bacterial types was done using the BIOLOG system and fatty acid profiling. Bacterial counts were analyzed using the restricted maximum likelihood methods (Genstat 5) and Wald statistics.

At the seedling stage, no interactions were detected and significant differences were observed for soil type, pea genotype and plant part (root-lower stem). The highest colonization at the seedling stage for each factor considered independently was detected in light clay, cv. Twiggy, and in roots. At the flower stage, the interactions plant genotype x plant part (pooled samples) and location x plant part (individual samples) were significant. There were no significant differences between locations or pea genotypes. Light-clay soil gave the highest counts in roots and stems. JI2202 was the highest colonized pea genotype at the flower stage. Populations in roots or lower stems were significantly higher than in upper stems. An increased average level of endophytes was found at the flower stage (from log 5.14 CFU/g at seedling stage to log 5.56 at flower stage).

A total of 570 isolates was used for bacterial species identification by the BIOLOG system and the fatty acid profiling. Gram-negative endophytic bacteria represented 90%, although there was an increase in the Gram-positive population at the flower stage, particularly in the light clay soil (from 3% to 20%). The dominant species were *Pseudomonas* sp. (54% of the total bacterial population), *Pantoea agglomerans* (11%) and *Stenotrophomonas maltophilia* (8%). *Arthrobacter* sp. and *Curtobacterium* sp. were the main Gram-positive species (40% and 17% respectively of the Gram-positive population). *Bacillus* sp. were poorly represented. Within a location, some species were only detected in certain cultivar(s). *Arthrobacter* sp., *Ps. viridiflava* and *Ps. syr.* pv. *syringae* were mainly detected at the flower stage and confined to the stems. *Ps. corrugata* was only detected at the seedling stage in the three field sites, while *Agrobacterium* sp. and *Acinetobacter* sp. were only detected at the flower stage. The ratio between the number of *Ps. fluorescens* in the root and the number in the stem (R/S) was *ca* 1 for sandy and heavy-clay soils and *ca* 2 for light-clay soil.

Induction of L-form cells (cell wall-less) of *Ps. syr.* pv. *pisi*

For the potential use of the L-form of *Ps. syr.* pv. *pisi* as a biocontrol agent of pathogenic *Ps. syr.* pv. *pisi*, an efficient production of L-form cells *in vitro* is an essential step. The first induction procedure was based on the induction method described by Paton and Innes (1991) for *Ps. syr.* pv. *phaseolicola*. Bacterial suspensions were shaken overnight in a liquid induction medium with penicillin (5000 units/ml, LIM+P), transferred to a solid medium without penicillin (SIM-P) and then a series of transfers to a solid medium with penicillin (SIM+P). Cell-wall deficient forms could be induced in LIM+P and granular and strongly vacuolated cells grew on solid medium. However, poor growth was observed in subsequent transfers and there was a reversion to the wild type. To overcome these limitations, new procedures were introduced to the induction protocol, including a viability check. Optimum induction time in LIM+P was lower for *Ps. syr.* pv. *pisi* than for *Ps. syr.* pv. *phaseolicola*. Viability of L-forms on SIM+P was low and decreased with time. Number of wild types increased in subsequent transfers indicating an increase of resistance to penicillin. Evaluation of a range of concentrations of ticarcillin combined with clavulanic acid and penicillin by the spiral gradient endpoint showed that the final inhibitory concentration on SIM+P for *Ps. syr.* pv. *pisi* was about four times lower than for *Ps. syr.* pv. *phaseolicola*. The use of ticarcillin combined with clavulanic acid provided an inhibition of the resistance. The method recommended is an induction on LIM+P (5000 units/ml) for 6h, then transfer to SIM-P which seems to be necessary for adaptability, and transfers to SIM+Ticarcillin.

CONCLUSIONS AND PROSPECTS

A breeding programme for resistance to pea bacterial blight must consider three major factors: (1) the distribution, frequency and aggressiveness of existing races, (2) the differential expression of resistance in different plant parts and (3) the host genetic resources. Race specific resistance genes are mainly dominant, easy to handle and generally confer a complete resistance. It has been shown in this study that this is true in the case of stem resistance, however, differential responses have been seen in leaf and/or pod for the expression of resistance genes R3 or R4 individually. The presence of R3+R4 has an additive effect and confers some resistance in both the leaf and pod. Race non-specific resistance present in *P. abyssinicum* confers a quantitative resistance to all races of *Ps. syr.* pv. *pisi*. However, as with race specific resistance, there is a shift in the resistant responses in leaf and/or pod, and resistance in the leaf to races 1, 3, 4 and 6 is not conferred in a consistent manner. Under field conditions, susceptibility in the leaf to race 6 is relatively low and susceptibility to race 4 is moderate in the leaf with some stem snapping. Race 4 is known to be a very aggressive race, and both race specific and race non-specific resistance seem not to be fully effective. There is some evidence that *P. abyssinicum* may also be carrying the race specific gene R2. An additional benefit from the use of *P. abyssinicum* is its frost tolerance, since frost damage is a factor that favours infection. With the pea genetic resources now available, possibilities for achieving a durable resistance include the combination of race non-specific resistance, which confers stem resistance to all races including race 6, and leaf resistance to races 2, 5 and 7; and the race specific genes R3+R4 for leaf resistance to races 1, 3, and 4 and their apparent enhancement of stem resistance to race 6.

The present study resulted in the development of: (1) F5s carrying race non-specific resistance derived from crosses between two *P. sativum* cultivars and two *P. abyssinicum* accessions, (2) progenies from the crosses of these F5s with cultivars carrying race specific resistance (genotypes R1, R3, R2+R3, R2+R3+R4) and (3) molecular markers for race non-specific resistance and its linkage to the classical markers *r* (wrinkled seed), *pa* (foliage colour) and *det* (determinate growth).

For developing a biological control programme with bacteria, it is essential to know the biology of the introduced bacterium agent and its interaction with the host. Research was focused on endophytic bacteria, an important group of plant associated bacteria. Representatives of this group are expected to have better traits for stable colonization of the host than epiphytic bacteria which are traditionally studied for biocontrol effects. Detection techniques for endophytic bacteria in pea were developed in this study, and their natural incidence under field conditions analyzed. Population levels were mainly in the range 10^4-10^5 CFU/g plant fresh weight). A cultivar effect

on population levels was demonstrated in two field trials during two different years. Studies on the mature stage of the crop were more complex due to increased interactions between the involved factors. The root and the lower part of the stem were consistently more colonized than the upper part of the stem and there was a increase of populations during the growing season. The light clay soil with the lowest humus content generated the highest endophytic populations.

The predominance of endophytic *Pseudomonas* sp. seems to be related to temperature, with a preference for cool conditions. Since endophytic bacterial populations have specificity for plant genotype, soil type and growth stage of the crop, it is important to realize that isolates with a biocontrol effect under certain growth conditions might not work under other conditions. Specialization of bacterial endophytes is related not only to the niche they colonize but also to their point of entrance. Although the root is considered to be the main entrance from which the endophytes can spread into other plant parts, bacterial endophytes (*Pantoea agglomerans*, *Ps. viridiflava*, *Ps. syringae*) reported as common epiphytes of the aerial parts of the plant will also enter the plant directly through wounds and natural openings in leafs and stems. The data on endophytic colonization of pea genotypes show that endophytic colonization levels maintain sufficiently high from seedling to mature stage. The large biodiversity of endophyte types opens the possibility to select for biocontrol strains to control *Ps. syr.* pv. *pisi* by antagonism, competition in the parenchymatic tissue of the stem or by induced resistance.

The development of an integrated programme for the sustainable control of pea bacterial blight would include selection for race specific and race non-specific resistance, using standard pathogenicity tests for race specific genes and molecular markers for race non-specific resistance together with screening for the selection of specific endophytic bacteria known to colonize with higher populations than 10^4 CFU/g, under field conditions and with different soil types. The combined use of these systems is expected to improve the durability of the resistance considerably.

Samenvatting

Bacteriebrand van erwt (*Pseudomonas syringae* pv. *pisi*) komt wereldwijd voor, met name in gebieden met koele en natte omstandigheden. Hoewel de ziekte van matig belang wordt beschouwd kan als gevolg ervan de gehele oogst verloren gaan. Ernstige schade treedt vooral op als bij in de winter gezaaid gewas de aantasting reeds in het kiemplantstadium plaatsvindt. Maatregelen om de ziekte te beheersen zijn gericht op het gebruik van ziektevrij zaad en de ontwikkeling van resistente cultivars. De mate van vóórkomen van specifieke races van *Ps. syr.* pv. *pisi* varieert per regio en er lijkt een negatieve correlatie op te treden met het voorkomen van de race-specifieke resistentiegenen. Voor race 6, een race waarvoor geen resistente cultivars beschikbaar zijn, wordt een toenemende incidentie gerapporteerd. Het ontwikkelen van alternatieve bestrijdingsmethoden en het integreren van bestrijdingsmethoden zijn nodig voor een effectievere en duurzamere beheersing van deze ziekte. In dit onderzoek zijn twee benaderingen onderzocht: (1) via veredeling, gebaseerd op de niet-specifieke resistentie aanwezig in *Pisum abyssinicum*, op basis waarvan stengelresistentie tegen alle races van het pathogeen verkregen kan worden en de wijze van overerving en inkruising van deze niet-specifieke resistentie in commerciële cultivars van *P. sativum* in combinatie met race-specifieke resistentie. (2) via een biologische aanpak, gebaseerd op onderzoek naar de ecologie van endofytische bacteriën van erwt en het potentiële gebruik ervan als biologische bestrijdingsagentia van bacteriebrand van erwt.

GENETISCHE RESISTENTIE

Resistentie tegen bacteriebrand van erwt in Spaans veredelingsmateriaal van *Pisum sativum*

Spaans veredelingsmateriaal van *P. sativum* werd getoetst om de frequentie van race-specifieke resistentie (R) genen tegen bacteriebrand van erwt en potentieel nieuwe bronnen van resistentie op te sporen. De toetsing werd uitgevoerd met zeven *Ps. syr.* pv. *pisi* races voor tien landrassen afkomstig van verschillende geografische gebieden die representatief waren voor de traditionele teelt van erwten in Spanje. Resistente fenotypes, zoals voorkomend in de verschillende landrassen, kwamen hoofdzakelijk overeen met de reeds eerder beschreven fenotypes in erwten cultivars gebaseerd op de gevestigde genotypen R3, R2+R3, R3+R4, R2+R3+R4. Een uitzondering werd gevonden voor landras ZP-0109; een heterogeen mengsel van diverse genotypen met partiële resistentie tegen race 6 en aanwijzingen voor het aanwezig zijn van de nog slecht gedocumenteerde R-genen, R5 en/of R6. R3 was het meest voorkomende resistentiegen dat in alle landrassen werd aangetroffen. R4 was aanwezig in vier en R2

in drie van de onderzochte landrassen. Geen van de landrassen was volledig vatbaar of resistent voor alle races.

Aanvullend is de genetische variatie voor resistentie tegen *Ps. syr.* pv. *pisi* vergeleken met de variatie voor vijf morfologische eigenschappen die weinig beïnvloed worden door omgevingsfactoren: bloemkleur, primaire kleur van het zaad, vorm van het zaad, mate van kromming van de peul en de vorm van het uiteinde van de peul. De variatie voor resistentie binnen een landras was in het algemeen laag (minder dan 10% voor zeven van de landrassen). De landrassen met de meeste variatie in resistentie gaven ook de meeste variatie in morfologische eigenschappen te zien. Landras ZP-0109 bleek voor de morfologische eigenschappen en de resistentie tegen bacteriebrand van erwt de grootste variatie te geven. Dit onderzoek is tevens de eerste gepubliceerde beschrijving van het voorkomen van *Ps. syr.* pv. *pisi* in Spanje en was gebaseerd op een collectie van 16 isolaten van erwt uit noordwest Spanje (Valladolid). Acht daarvan werden geïdentificeerd als race 6 en acht als race 4.

Niet-specifieke resistentie tegen brandvlekkenziekte van erwt afkomstig van *Pisum abyssinicum*

De overerving van potentiele niet-specifieke resistentie tegen bacteriebrand van erwt afkomstig van *P. abyssinicum* werd onderzocht via kruisingen tussen twee *P. sativum* cultivars ('Kelvedon Wonder' en 'Fortune') en twee *P. abyssinicum* accessies (JI2202 en JI1640). ''K. Wonder'' is vatbaar voor alle races van *Ps. syr.* pv. *pisi*. ''Fortune'' is resistent tegen alle races behalve race 6 (bevat race-specifieke genen R2, R3 en R4) en JI2202 en JI1640 zijn resistent of partiëel resistent voor alle races inclusief race 6. Onderzoek naar resistentie in de nakomelingen werd uitgevoerd door stengelinoculaties met race 6.

Alle planten van de F1 generatie waren overeenkomstig de verwachting vatbaar voor het pathogeen. Er waren reciproke verschillen in de productie van nakomelingen, deze was lager voor de kruisingen waarbij *P. abyssinicum* als vrouwelijke ouder gebruikt werd. De uitsplitsingsverhouding in de F2 afkomstig van kruisingen met 'K. Wonder' waren overwegend 1:3 Resistent:Vatbaar (R:S), ($P>0.05$). De uitsplitsingsverhouding voor het morfologische kenmerk hoogte van de plant was zoals verwacht 3:1 Hoog:Dwerggroei (T:D), ($P>0.5$). Het resultaat van de kruising JI2202 x 'K. Wonder' vormde een uitzondering, waarbij de uitsplitsing niet overeenkwam met de 1:3 R:S noch met de 1:3 T:D en de vruchtbaarheid van de F1 lager was. De F2s afkomstig van de kruisingen met 'Fortune' waren enigszins verschillend door een hoger aantal resistente planten dan in de kruisingen met 'K. Wonder'.

De uitsplitsingspatronen van de F3 kwamen niet overeen met de verwachting op

basis van een enkel recessief gen. F3 planten verkregen van geselecteerde resistente en vatbare F2s splitsten uit in een complex patroon: drie uitsplitsingen (13:3, 1:1, 3:13 R:S) voor F3s afkomstig van resistente F2s en twee uitsplitsingen (1:1, 3:13 R:S of uniforme vatbaarheid) voor F3s afkomstig van vatbare F2s. Voor de F3s van niet geselecteerde F2s werden dezelfde uitsplitsingsklassen gevonden, behalve dat hierbij ook twee groepen gevonden werden die uniform resistent waren. Het relatief lage aantal planten in deze groepen sluit een 13:3 verdeling niet uit. De drie waargenomen uitsplitsingsverhoudingen waren voor beide data sets aanwezig in de nakomelingen van dezelfde F1 planten.

F5 families werden verkregen door kruisingen met resistente selecties van de F2 en F3 op basis van het relatief veel voorkomen van resistente planten in de F3 families. Op het moment dat deze selectie werd gemaakt was nog niet bekend dat deze families uitsplitsten in een 13:3 R:S in de F3. Zevenentwintig van de dertig families waren uniform resistent. Geselecteerde resistente planten van de F5 met een duidelijke resistente reactie van de plant (zonder symptomen van waterverzadiging in het weefsel) werden gekruist met de ouderlijke lijnen en met de cultivars Solara (met race-specifiek resistentiegen R3), Belinda (R3), Cea (R3), Esla (R3), Frisson (R2 en R3), 'Fortune' (R2, R3 en R4), Ballet and Orb (resistentie onbekend) en met de accessie JI15 (R1).

De aanwezigheid van race-specifieke genen in 'Fortune' tegen alle races van *Ps. syr.* pv. *pisi* behalve race 6 scheen de resistentie tegen race 6 te verhogen in kruisingen met *P. abyssinicum*. Deze waarneming is gebaseerd op de relatieve overmaat aan resistente planten in de F2 in vergelijking met kruisingen met K.Wonder. De enige twee families met een blijkbaar uniforme resistentie in de F3 waren beide afkomstig van kruisingen met 'Fortune'.

Er werd in de kruisingen een mogelijke koppeling waargenomen tussen resistentie en dwerggroei. Het aantal planten met deze koppeling in de F2 was hoger dan verwacht mocht worden op basis van een onafhankelijke uitsplitsing. Echter voor maar één kruising (JI2202 x 'Fortune') was deze koppeling significant. Voor F3s afkomstig van geselecteerde F2s, waren alle planten, behorend bij de F3 families in de 13:3 R:S categorie, óf uniform met dwerggroei óf uitsplitsend voor lengte, maar geen enkele familie bevatte alleen maar lange planten. Bij de F5 vertoonden 22 van de 27 uniform resistente families tevens uniforme dwerggroei.

Vanwege de complexheid van de uitsplitsingsverhoudingen werd een moleculaire aanpak gekozen om de mogelijke bronnen voor overerving op te sporen. Daarbij werd gebruik gemaakt van een *Tyl-copia* retrotransposon van erwt (PDR1) en sequence specific amplification polymorphism merkers (SSAP). De amplificatieproducten van *P. sativum* ouderlijnen bleken overeen te komen met die van een goed gekarakteriseerde *P. sativum* cultivar (JI399), terwijl de twee *P. abyssinicum* lijnen

identiek waren aan de eerder gekarakteriseerde *P. abyssinicum* accessies. Onderzoek naar deze merkers in de uitsplitsingen van de F3, F4 en F5 populaties was gericht op individuele homozygote planten die resistent of vatbaar waren.

Er werden drie co-segregerende merkers met resistentie en drie met vatbaarheid werden gevonden. Alle planten die wel de drie *P. abyssinicum* merkers bezaten maar de *P. sativum* merkers misten waren resistent. Deze planten waren grotendeels afkomstig van resistente selecties van de 13:3 R:S F3 klasse. De helft van de planten met enkele van de *P. sativum* merkers was afkomstig van de 1:1 of 3:13 R:S klassen. De mate van koppeling werd geschat op basis van een enkele groep van merkers (enkel recessief gen) en de recombinatie frequenties werden bepaald door de F2 en RI modellen te hanteren als boven en ondergrenzen. Deze analyse geeft aan dat deze zes merkers vermoedelijk maximaal 25 cM van de veronderstelde resistentie factoren verwijderd zijn. Het is echter mogelijk dat enkele van de fenotypische waarnemingen zijn beïnvloed door modificerende loci. Twee van de *P. sativum* merkers werden met behulp van de nakomelingen van twee recombinante terugkruisingen (JI15 x JI399 en JI281 x JI 399) gelokaliseerd op koppelingsgroep V. De afstand tussen deze twee merkers was moeilijk te bepalen als gevolg van de verschillende recombinatie frequenties van de twee kruisingen, maar bedraagt waarschijnlijk 20 cM. De allelen *r* (gerimpeld zaadoppervlak), *pa* (bladkleur) of *det* (bepalend voor groeitype) konden worden gebruikt als klassieke merkers voor de inkruising van de resistentie eigenschap.

Gebaseerd op de hiervoor beschreven resultaten is het voorgestelde overervingsmodel gebaseerd op een belangrijk recessief gen aanwezig in *P. abyssinicum* en een set van waarschijnlijk twee modificerende genen die aanwezig kunnen zijn in *P. abyssinicum* of *P. sativum*.

Reacties op bacteriebrand van erwt in verschillende delen van de plant: kas- en veldonderzoek.

De toetsing op resistentie tegen bacteriebrand van erwt is als regel uitgevoerd via inoculatie van de stengel, waarbij aangenomen wordt dat de reactie vergelijkbaar is met die in andere delen van de plant. Echter, stengelinoculaties die vlak bij de stipulae werden uitgevoerd met race 3 op een cultivar met R3, lieten soms een vatbare reactie (waterverzadigde vlekken) zien die zich uitbreidde in de stipulae, terwijl de stengel ook de verwachte necrotische (resistente) reactie gaf. Op basis daarvan is uitgebreider onderzoek gedaan naar de verschillende reacties van de zeven races in stengels, bladeren en peulen. De experimenten werden in de kas uitgevoerd voor een reeks erwten accessies (*P. sativum* en *P. abyssinicum*) die in het bezit waren van één enkel

race-specifiek gen, verschillende combinaties van race-specifieke genen, of niet-specifieke resistentie.

Uit deze experimenten bleek dat een resistente reactie in de stengel niet altijd overeenkwam met resistentie in het blad of de peul. Dit verschijnsel trad zowel bij race-specifieke als bij niet-specifieke reacties op. De race-specifieke genen gaven een consistente expressie van de resistentie in de stengel, maar gaven een variabele reactie in het blad en de peul, afhankelijk van de aanwezigheid van compatibele combinaties van het R-gen en het A-gen (avirulentie gen van het pathogeen). Race-specifiek gen R2 gaf in het algemeen resistentie tegen races 2, 5, en 7 (elk met A2 gen) in alle delen van de plant. De afzonderlijke genen R3 en R4 gaven meestal beperkte blad- en peulresistentie, maar de combinatie verhoogde de resistentie voor symptomen in blad en peul. Accessies met een niet-specifieke resistentie waren resistent tegen de races 2, 5 en 7 in alle delen van de plant, de races 1, 3, 4 en 6 gaven een consistente stengelresistentie maar een variabele reactie in het blad en de peul. Dit geeft aan dat niet-specifieke resistentie niet altijd tot uiting komt in het blad en de peul en dat het R2 gen ook kan voorkomen in JI2202 en JI1640.

Aanvullend is het effect van omgevingsfactoren op de veldexpressie van de resistentie bestudeerd in het najaar en de winter van 1998-99. Het experiment werd uitgevoerd met in potten opgekweekte planten van 'K. Wonder', JI2202 en JI1640 die geïnoculeerd waren met de belangrijkste races (2, 4 en 6). Om een natuurlijke infectie zo goed mogelijk te simuleren werden de planten besproeid met een bacteriesuspensie zonder dat ze vooraf verwond waren. Acht weken na de inoculatie waren alle 'K. Wonder' planten dood, maar *P. abyssinicum* planten waren in goede conditie, hoewel bij de met race 4 geïnoculeerde planten soms stengeldeformatie optrad. Vorstschade staat bekend als een factor die de infectie stimuleert en deze schade was ernstiger in K.Wonder dan in *P. abyssinicum* De vorsttolerantie in *P. abyssinicum* vormt een complementaire factor voor ziekteonderdrukking in wintergezaaide gewassen. Race 4 was het meest agressieve race zowel in de kas als onder veldcondities. De vatbaarheid van *P. abyssinicum* bladeren voor race 6 was onder veldomstandigheden minder dan in de kas. Dit geeft aan dat de combinatie van niet-specifieke resistentie van *P. abyssinicum* met de race-specifieke genen R3+R4 een zeer goede bescherming kunnen bieden, zelfs voor wintergeteelde gewassen.

MOGELIJKHEDEN VOOR BIOLOGISCHE BESTRIJDING GEBASEERD OP DE ECOLOGIE VAN ENDOFYTISCHE BACTERIËN

Detectie van natuurlijk voorkomende endofyten in cultivars van erwt

Het natuurlijk voorkomen van endofytische bacteriën in de stengels van erwt en methoden voor de isolatie ervan zijn bestudeerd in een rassenproef in het zuiden van Nederland. Een snelle screeningstechniek voor de detectie van natuurlijke endofyten werd ontwikkeld op basis van afdrukken van het oppervlak van dwars doorgesneden stengels op 5% TSA (Trypticase Soya Agar) nadat de stengels vooraf gedesinfecteerd waren met 1% beschikbaar chloor met 0,1% Tween 80 gedurende 5 min. In het bloeistadium werden de stengels van elf cultivars van de basis naar de top onderzocht. De afdrukken van de dwarsdoorsneden van de stengel gaven een semi-kwantitatieve bepaling van de populatie niveaus en zijn gebruikt voor het verkrijgen van isolaten van endofytische bacteriën. Voor vijf cultivars werd endofytische kolonisatie vastgesteld, waarbij cultivar 'Twiggy' de hoogste en meest consistente kolonisatie liet zien die significant hoger lag dan die van de andere cultivars. Aan cv. 'Twiggy' is met deze stempelmethode verder onderzoek gedaan door 20 planten in het peulstadium van stengelbasis tot top te analyseren. Stengeldelen van het 3e en het 4e internodium zijn na uitwendige ontsmetting gehomogeniseerd en met de spiralplater uitgeplaat op drie media (5% TSA voor heterotrofe, R2A voor oligotrofe en SC voor moeilijk kweekbare bacteriën). Het aantal endofytische bacteriën nam toe van de basis naar de top. De populaties varieerden van 10^3 tot 10^7 CFU/g vers stengelgewicht, maar waren gewoonlijk tussen 10^4 en 10^5 CFU/g. De populaties in één van de blokken verschilde significant van die van de andere blokken. De recovery van endofyten in een vergelijking van de drie media voor een serie van 30 stengelextracten gaf voor TSA een recovery efficiëntie die significant hoger (17%) was dan voor R2A en SC. De dominante soorten waren *Pantoea agglomerans* en *Pseudomonas fluorescens*. *Ps. viridiflava* was vooral aanwezig in één van de blokken, terwijl *Bacillus megaterium* uitsluitend in dit blok werd gevonden. De efficiëntie en betrouwbaarheid van de stempelmethode is vergeleken met de traditionele uitplaatmethode. De positieve correlatie tussen het aantal kolonies in stempelafdrukken en het aantal in, met de spiralplater verwerkte monsters gaf aan dat de stempelmethode op agar een goede methode is voor semi-kwantitatieve bepalingen in stengels van erwt die 10^4 tot 10^7 CFU/g versgewicht bevatten.

Factoren die de endofytische populaties van bacteriën in erwt beinvloeden

Op basis van de hiervoor beschreven resultaten lijkt het genotype van de plant een rol te spelen bij de fenotypische kolonisatie van erwt. Verdere studies hiernaar zijn gecombineerd met onderzoek naar de relatie tussen bodemtype, groeistadium van het gewas en naar de endofytische kolonisatie van verschillende plantendelen. Dit onderzoek werd uitgevoerd in drie gebieden ten oosten van het centrum van Nederland

met bodems van een verschillend type (textuur, organische stof en pH). Vier cultivars van *P. sativum* met verschillende commerciële toepassingen zijn onderzocht: Solara, Norli, 'Fortune' (ouderlijn in het veredelingsprogramma) en 'Twiggy' (beste door endofyten gekoloniseerde cultivar) en een accessie van *P. abyssinicum* JI2202 (ouderlijn in veredelingsprogramma). Op iedere locatie waren twee velden met twee blokken per veld en vijf subblokken per blok, overeenkomend met een complete blokkenproef. De planten zijn bemonsterd in het zaailing- en het bloeistadium. Wortel- en stengeldelen zijn individueel verwerkt of na samenvoeging van 5 planten. De plantdelen zijn na oppervlakkige ontsmetting via spiralplating uitgeplaat op 5% TSBA. De karakterisering van de bacterietypen is uitgevoerd met het Biolog systeem en of via vetzuur profilering. De bacterietellingen zijn geanalyseerd met Genstat 5 (maximum likelihood methode) en met Wald statistics.

De aantallen endofyten in de bepalingen van de samengevoegde monsters waren hoger dan voor individueel verwerkte monsters, speciaal in het zaailingstadium. In het zaailingstadium traden nog geen interacties tussen de onderzochte factoren op. Significante verschillen werden gevonden voor de effecten van bodemtype, het genotype van de erwt en de kolonisatie van de wortel en de stengelbasis. De hoogste kolonisatie werd gevonden in de wortelbasis van cv. 'Twiggy' in lichte kleigrond. In het bloeistadium werden significante interacties gevonden tussen genotype en plantendeel en locatie en plantendeel. Er werden geen significante verschillen meer gevonden tussen locaties en genotypen. In het algemeen werd de hoogste endofytische kolonisatie gevonden in lichte kleigrond. De accessie van *P. abyssinicum* JI2202 was het hoogst gekoloniseerd in het plantstadium. Populaties in de wortel- en stengelbasis waren significant hoger dan in het middendeel van de stengel. Vergeleken met het zaailingstadium was in het bloeistadium het gemiddelde niveau enigszins toegenomen (5,14 CFU naar 5,56 CFU per gram versgewicht).

In totaal zijn 570 van de bacterieisolaten voorlopig geïdentificeerd m.b.v. vetzuuranalyse en/of Biolog. Voor beide plantstadia was 90% van de isolaten Gram-negatief. De Gram-positieve bacteriën namen toe in het bloeistadium met name in de lichte kleigrond (van 3% naar 20%). De dominante soorten waren *Pseudomonas* sp. (54% van de totale populatie), *Pantoea agglomerans* (11%) en *Stenotrophomonas maltophilia* (8%). *Arthrobacter* en *Curtobacterium* waren de meest voorkomende gram positieve typen (respectievelijk 40% en 17% van de Gram positieven). *Bacillus* sp. kwamen weinig voor. Binnen één locatie werden bepaalde endofyt-typen alleen gevonden in bepaalde cultivar(s). *Arthrobacter* sp., *Ps. viridiflava* en *Ps. syringae* pv. *syringae* werden voornamelijk gevonden in de stengels in het bloeistadium. *Ps. corrugata* werd op alle drie de lokaties alleen gevonden in het zaailingstadium, terwijl *Agrobacterium* en *Acinetobacter* sp. alleen in het bloeistadium werden gevonden. De verhouding van het aantal *Ps. fluorescens* in de wortel t.o.v. de stengel (R/S) was ca. 1

voor zandgrond en voor zware klei en ca. 2 voor lichte klei.

Inductie van L-vorm cellen (celwandvrij) van *Ps. syr.* pv. *pisi*

Een essentiële stap voor het gebruik van L-vorm cellen van *Ps. syr.* pv. *pisi* voor de biologische bestrijding van *Ps. syr.* pv. *pisi* is een efficiënte productie van L-vorm cellen *in vitro*. De eerst getoetste procedure was gebaseerd op de inductiemethode voor *Ps syr.* pv. *phaseolicola* zoals gebruikt door Paton and Innes (1991). Bacteriesuspensies werden de overnacht geschud in een vloeibaar inductiemedium met penicilline (5000 units/ml. LIM+P), overgeënt naar een vast medium zonder penicilline (SIM-P) en vervolgens een aantal malen overgezet op vast medium met penicilline (SIM+P). Celwand deficiënte vormen konden worden geïnduceerd in LIM+P terwijl granulaire cellen en cellen met grote vacuolen werden gevormd op vast medium. In de daaropvolgende overentingen was de groei slecht en werd een terugval naar cellen van het wildtype gevonden. Om deze problemen op te lossen zijn modificaties op het inductieprotocol onderzocht en zijn vitaliteitcontroles van de cellen uitgevoerd. De optimale inductietijd in LIM+P was lager voor *Ps. syr.* pv. *pisi* dan voor *Ps. syr.* pv. *phaseolicola*. De vitaliteit van L-vorm cellen op SIM+P was laag en nam in de tijd af. Het aantal wildtype cellen nam toe in de opeenvolgende overzettingen, wat aangaf dat er een toename van de resistentie tegen penicilline optrad. Evaluatie van de inductie van L-vorm cellen met de z.g. spiralplate endpoint methode voor een reeks concentraties van penicilline en de combinatie van ticarcilline en clavulaanzuur toonde aan dat de hoogst remmende concentratie op SIM+P voor *Ps. syr.* pv. *pisi* ongeveer een factor vier lager was dan voor *Ps. syr.* pv. *phaseolicola*. Het gebruik van ticarcilline in combinatie met clavulaanzuur gaf een onderdrukking van de vorming van resistentie cellen. De aanbevolen methode is gebaseerd op een inductie in LIM+P (5000 units/ml) gedurende 6 uur, overenten naar SIM-P voor aanpassing van de cellen, gevolgd door overenting naar een vast inductiemedium met de combinatie van ticarcilline en clavulaanzuur.

CONCLUSIES EN VOORUITZICHTEN

Een veredelingsprogramma voor resistentie tegen bacteriebrand van erwt vereist kennis van drie belangrijke aspecten: (1) kennis over de verspreiding, mate van voorkomen en agressiviteit van de bestaande races, (2) kennis over de verschillen in de expressie van de resistentie in verschillende plantendelen en (3) beschikbaarheid van genetische bronnen van de waardplant. Race-specifieke resistentiegenen zijn overwegend dominant, eenvoudig toe te passen en geven als regel een volledige

resistentie. Dit onderzoek toonde aan dat dit opgaat voor de stengelresistentie, maar voor resistentie in bladeren en of de peulen werden voor de individuele genen R3 en R4 wisselende reacties waargenomen. De aanwezigheid van R3+R4 genen had een additief effect en droeg bij aan de resistentie in zowel het blad als de peul. Niet-specifieke resistentie zoals aanwezig in *P. abyssinicum* droeg bij aan een kwantitatieve resistentie tegen alle races van *Ps. syr.* pv. *pisi*. Echter overeenkomstig de situatie bij race-specifieke resistentie, was er ook hierbij een verschuiving opgetreden in de resistente reactie in blad en/of peul. De resistentie in het blad voor races 1,3,4 en 6 werd niet op een consistente wijze overgedragen. Onder veldcondities is de vatbaarheid van het blad voor race 6 relatief laag en voor race 4 matig; wel zijn voor race 4 soms stengeldeformaties gevonden. Van race 4 is bekend dat het een erg agressief race is waartegen de race-specifieke en de niet-specifieke resistentie niet volledig afdoende zijn. Er zijn enkele aanwijzingen dat *P. abysinicum* ook het race-specifieke gen R2 kan bevatten. Een bijkomend voordeel van het gebruik *van P. abyssinicum* is de vorsttolerantie, omdat vorstschade het infectieproces bevordert. Met de nu beschikbare genetische bronnen van resistentie in erwt zijn er, inclusief de toepassing van niet-specifieke resistentie, goede mogelijkheden voor het verkrijgen van een duurzame resistentie. De niet-specifieke resistentie geeft voor alle races stengelresistentie en voor races 2,5 en 7 bladresistentie, terwijl de race-specifieke genen bladresistentie tegen races 1, 3 en 4 en een blijkbaar verhoogde resistentie tegen race 6 geven.

Dit onderzoek heeft geresulteerd in de ontwikkeling van (1) F5s met niet-specifieke resistentie afkomstig van kruisingen tussen twee *P. sativum* cultivars en twee *P. abyssinicum* accessies, (2) nakomelingen van de kruisingen van deze F5s met cultivars met race-specifieke resistentie (genotypen R1, R3, R2+R3, R2+R3+R4). en (3) moleculaire merkers voor niet-specifieke resistentie en de relatie ervan met de klassieke merkers *r*, *pa* en *det*. De *P. abyssinicum* accessie JI2385, de enige bekende *P. abyssinicum* accessie die vatbaar is voor race 6, kan worden gebruikt voor verder onderzoek naar de overerving van resistentie afkomstig van *P. abyssinicum*. Kruisingen tussen JI2385 en *P. abyssinicum* accessies met niet-specifieke resistentie kunnen worden gebruikt om duidelijkheid te krijgen of de complexe F3 verhoudingen in de kruisingen tussen *P. sativum* en *P. abyssinicum* het gevolg zijn van de verdere genetische achtergrond (e.g. via modificerende genen) of mede het gevolg zijn van een bepaalde mate van genomische incompatibiliteit.

Voor het ontwikkelen van een biologisch bestrijdingprogramma met bacteriën is het van groot belang de biologie van de geïntroduceerde bacterie en de interactie ervan met de waardplant te kennen. Het onderzoek was gericht op de endofytische bacteriën, een nog weinig onderzochte groep van plant-geassocieerde bacteriën. Van vertegenwoordigers van deze groep wordt verwacht dat ze betere eigenschappen

bezitten voor een stabiele kolonisatie van de waardplant dan de epifytische bacteriën waarnaar traditioneel veel onderzoek wordt uitgevoerd. In dit onderzoek zijn detectiemethoden voor endofytische bacteriën in erwt ontwikkeld en is hun natuurlijk voorkomen in het veld bestudeerd. De populatieniveaus lijken voldoende hoog (gemiddeld 10^4 tot 10^5) om een bestrijdingseffect te verwachten. Een effect van de cultivars op de populatieniveaus werd gevonden in twee veldexperimenten uitgevoerd in twee verschillende jaren. Analyse van de endofytische kolonisatie in het bloeistadium bleek veel ingewikkelder door het optreden van interacties tussen de betrokken factoren. De wortel en de stengelbasis waren hoger gekoloniseerd dan het bovendeel van de stengeldelen. Er werd een geringe toename van de populaties gedurende het groeiseizoen geconstateerd. De lichte kleigrond met de laagste humusconcentratie gaf de hoogste endofytische kolonisatie.

De dominantie van endofytische *Pseudomonas* sp. kan deels worden verklaard uit de voorkeur van deze groep voor wat koelere omstandigheden. Omdat endofytische bacteriën specifiek kunnen zijn voor bepaalde gewassen en beïnvloed kunnen worden door het genotype, bodemtype en plantstadium is het mogelijk dat bepaalde ziektewerende endofyten onder bepaalde condities wel werken en onder andere niet. Succesvolle kolonisatie door nuttige endofyten is niet alleen gerelateerd aan de niche die ze koloniseren, maar ook aan de wijze van binnendringen. Hoewel de wortel beschouwd wordt als de belangrijkste plaats van binnendringen van waaruit de rest van de plant gekoloniseerd wordt, zijn er bepaalde endofyten gerapporteerd als algemene epifyten van de bovengrondse delen van de plant (*Pantoea agglomerans, Ps. viridiflava, Ps. syringae*) die de plant mogelijk direct kunnen binnengedrongen via wonden of natuurlijke openingen in het blad en de stengel. De gegevens over de endofytische kolonisatie van de erwtengenotypen tonen voor o.a. *Ps. fluorescens* aan dat de endofytische kolonisatie van zaailingen en bloeiende planten voldoende hoog zijn om effecten te verwachten. De grote biodiversiteit van de endofyt typen geeft de mogelijkheid te selecteren voor stammen voor de bestrijding van *Ps. syr.* pv. *pisi* op basis van antagonisme en competitie in het parenchymatische weefsel van de stengel of via geïnduceerde resistentie.

De ontwikkeling van een geïntegreerd programma voor de duurzame bestrijding van bacteriebrand van erwt zou gericht kunnen zijn op de selectie van race-specifieke en niet-specifieke resistentie, waarbij de pathogeniteitstoets gebruikt kan worden voor het screenen van race-specifieke genen en moleculaire merkers voor de niet-specifieke genen. Onderzoek naar ziektewerende nuttige endofytische bacteriën kan het best worden gericht op die typen endofyten die een goede kolonisatie van de belangrijkste cultivars geven onder voor de teelt relevante veldomstandigheden. Het gecombineerd toepassen van deze systemen zal naar verwachting de duurzaamheid van de resistentie in belangrijke mate verbeteren.

Resumen

La grasa bacteriana del guisante (*Pseudomonas syringae* pv. *pisi*) ocurre mundialmente, principalmente en condiciones templadas y húmedas. Aunque está considerada de moderada importancia, el cultivo entero se puede perder si la infección se produce en el estado de plántula, particularmente en los cultivos de siembra otoñal. Las medidas utilizadas hasta ahora para el control de la enfermedad han sido el usode semilla no portadora del patógeno y el uso de cultivares con genes de resistencia específica. La frecuencia de aparición de las razas de *Ps. syr.* pv. *pisi* varía geográficamente y parece existir una correlación negativa con la frecuencia de los genes de resistencia compatibles con los genes de avirulencia de una raza determinada. Se ha publicado el continuado aumento de la incidencia de la raza 6, para la que en la actualidad no existen cultivares resistentes. En el presente estudio dos enfoques para abordar el control de la enfermedad han sido investigados: (1) un enfoque genético, a través del estudio de la resistencia general o no-específica presente en *Pisum abyssinicum*, la cual confiere resistencia en el tallo a todas las razas del patógeno, su modo de herencia y la introgresión en cultivares comerciales de *P. sativum* en combinación con los genes de resistencia específica; (2) un enfoque referido al control biológico, a través del estudio de la ecología de las bacterias endófitas del guisante y su uso potencial como agentes de control biológico de la grasa bacteriana del guisante.

RESISTENCIA GENETICA

Resistencia a la grasa bacteriana del guisante en el germoplasma español de *Pisum sativum*

La resistencia a la grasa bacteriana del guisante en el germoplasma español de *Pisum sativum* fue investigada para dilucidar la frecuencia de los genes de resistencia específica (genes R) e identificar nuevas fuentes potenciales de resistencia. La investigación se llevó acabo mediante la inoculación de diez landraces originarios de diferentes zonas geográficas de España y considerados respresentativos del cultivo tradicional del guisante en España. Los fenotipos de resistencia de los diferentes landraces fueron principalmente como los descritos previamente en cultivares de guisante y correspondieron a los genotipos resistentes R3, R2+R3, R3+R4, R2+R3+R4. La excepción fue el landrace ZP-0109, una mezcla genética con resistencia parcial a la raza 6, y con cierta evidencia de la presencia de los genes R5 y/o R6, los cuales están escasamente documentados. El gen de resistencia más común fue R3, presente en todos los landraces. R4 estuvo presente en cuatro y R2 en tres de los landraces estudiados. Ninguno de ellos fue completamente susceptible o resistente

a todas las razas.

Adicionalmente, la diversidad genética para la resistencia a *Ps. syr.* pv. *pisi* se comparó con la diversidad para cinco carácteres morfológicos los cuales fueron seleccionados por ser en gran parte independientes de las influencias medio ambientales: color de la flor, color de la semilla, forma de la semilla, el grado de curvatura de la vaina y la forma de la punta de la vaina. La variación entre landraces para la resistencia fue generalmente baja (menos del 10% para siete de los landraces). Los mismos landraces que mostraron alta variación para la resistencia también mostraron una alta variación para los caracteres morfológicos. El landrace ZP-0109 fue muy diverso para todos los caracteres estudiados. Esta investigación también representa la primera publicación de la presencia de *Ps. syr.* pv. *pisi* en España basado en una colección de 16 aislamientos procedentes del Noroeste de España (Valladolid), ocho fueron identificados como la raza 6 y ocho como la raza 4.

Resistencia no-específica a la grasa bacteriana del guisante procedente de *Pisum abyssinicum*

La herencia de la potencialmente resistencia no-específica a la grasa bacteriana del guisante procedente de *Pisum abyssinicum* fue estudiada mediante un programa de cruzamientos entre dos cultivares de *P. sativum* ('Kelvedon Wonder' y 'Fortune') y dos accesiones de *P. abyssinicum* (JI2202 y JI1640). 'K. Wonder' es susceptible a todas las razas de *Ps. syr.* pv. *pisi*, 'Fortune' es resistente a todas las razas excepto a la raza 6 (posee los genes de resistencia específica R2, R3 y R4), y JI2202 y JI1640 son resistentes/ parcialmente resistentes a todas la razas, incluyendo la raza 6. El cribado para la resistencia de la descendencia se realizó mediante la inoculación en el tallo con la raza 6.

La primera generación (F1) fue generalmente susceptible como se esperaba. Hubo diferencias en los cruzamientos recíprocos referente a la fecundidad de las plantas de la F1, esta fue menor para los cruzamientos en los que *P. abyssinicum* fue el parental femenino. Los ratios de la segregación en la F2 derivada de los cruzamientos con 'K. Wonder' fueron pedominantemente 1:3 Resistente:Susceptible (R:S), ($P>0.05$). La segregación para la altura de la planta (marcador morfológico) fue 3:1 Porte alto:Porte bajo (T:D), ($P>0.5$) como se esperaba. La excepción fue el cruzamiento JI2202 x KW, el cual no segregó 1:3 R:S ni 3:1 T:D y también tuvo una menor fertilidad en la F1. Las F2s derivadas de los cruzamientos con 'Fortune' se comportaton de una manera un tanto diferente, con un número mayor de plantas resistentes que en los cruzamientos con 'K. Wonder'.

Los modelos de segregación en la F3 no correspondieron con los esperados para un

solo gen recesivo. Las plantas de la F3 producidas de las F2s seleccionadas como resistentes o susceptibles, segregaron en un complejo modelo de acuerdo a los siguientes ratios: tres categorías segregantes (13:3, 1:1, 3:13 R:S) para las F3s derivadas de las F2s resistentes y dos categorías segregantes (1:1, 3:13, R:S) o uniformemente susceptible para las F3s producidas de F2s suscecptibles. Para las F3s derivadas de las F2s que no fueron seleccionadas, se observaron la mismas categorías, excepto que en este caso dos familias fueron uniformemente resistentes, sin embargo el relativo bajo número de plantas no excluye una segregación 13:3. De ambos grupos de datos, los tres ratios de segregación estuvieron presentes en proles derivadas de las mismas plantas de la F1.

Las familias de la F5 se produjeron a partir de selecciones resistentes de la F2 y F3 basadas en la preponderancia de plantas resistentes en las familias de la F3, sin embargo cuando estas selecciones fueron hechas no se había reconocido todavía que estas familias segregaban de acuerdo al ratio 13:3 R:S. Veintisiete de 30 familias fueron uniformemente resistentes. Plantas resistentes de la F5 fueron seleccionadas en base a una marcada reacción resistente y a la no producción de 'water-soaking' y fueron cruzadas con las líneas parentales, con cultivares comerciales: Solara (con el gen de resistencia específica R3), Belinda (R3), Cea (R3), Esla (R3), Frisson (R2 y R3), 'Fortune' (R2, R3 y R4), Ballet y Orb(resistencia desconocida), y con la accesión JI15 (R1).

La presencia en 'Fortune' de los genes de resistencia específica, los cuales confieren resistencia a todas las razas de *Ps. syr.* pv. *pisi* excepto a la raza 6, parecieron intensificar la resistencia a la raza 6 en cruzamientos con *P. abyssinicum.* Esto se pudo observar en un exceso de plantas resistentes en la F2 comparado con los cruzamientos en los que 'K. Wonder' era una de las líneas parentales. Las únicas dos familias que aparentemente fueron uniformemente resistentes en la F3 derivaron de cruzamientos con 'Fortune'.

Se observó un aparente ligamiento del porte bajo a la resistencia. El número de plantas que fueron de porte bajo y resistentes en la F2 fue mayor que el esperado para una segregación independiente. Sin embargo, sólo para un cruzamiento (JI2202 x FORT) un valor significativo de la probabilidad estadística indicó la existencia de ligamiento. Para las F3s producidas de F2s que fueron seleccionadas, todas las plantas de F3s de la categoría 13:3 R:S fueron o de porte bajo o segregaron pero no fueron uniformemente de porte alto. En la F5, 22 de 27 familias uniformemente resistentes fueron uniformemente de porte bajo.

Debido a la complejidad de los modelos de segregación, se tomó un enfoque molecular para dilucidar el posible control genético. Para ello se utilizó un retrotransposón del guisante del grupo *Ty1-copia* (PDR1) y los marcadores SSAP ('sequence specific amplification polymorphism'). Los productos resultantes de la

amplificación correspondientes a las líneas parentales de *P. sativum* fueron similares a los obtenidos para el cultivar de *P. sativum* (JI399), previamente caracterizado a este estudio, mientras que las accesiones de *P. abyssinicum* fueron idénticas a otras accesiones de *P. abyssinicum* previamente caracterizadas. En el cribado de las poblaciones segregantes F3, F4 y F5 para la obtención de marcadores moleculares , se utilizaron plantas que fueran potencialmente homocigotas para la la resistencia o la susceptibilidad.

Se identificaron tres marcadores cosegregando con la resistencia y tres cosegregando con la susceptibilidad. Todas las plantas testadas en las que los tres marcadores procedentes de *P. abyssinicum* estuvieron presentes y los tres marcadores procedentes de *P. sativum* ausentes, fueron resistentes. Estas plantas principalmente derivaron de las selecciones resistentes pertenecientes a la categoria 13:3 R:S. Sin embargo, la mitad de las plantas en las cuales los marcadores derivados de *P. sativum* estuvieron presentes derivaron de las categorías 1:1 o 3:13 R:S. La intensidad del ligamiento se estimó considerando un solo grupo de marcadores (resistencia controlada por un solo gen recesivo) y las frecuencias de recombinación se estimaron tratando la F2 y la poblacion RI como modelos para los máximos y mínimos valores. Este análisis sugerió que los seis marcadores se encuentran a una distancia no mayor de aproximadamente 25 cM del locus que es considerado el principal determinante de la resistencia; sin embargo, es posible que algunas de las asignaciones dadas a los fenotipos estén influenciadas por loci modificadores. Dos de los marcadores derivados de *P. sativum* fueron mapeados a través de dos poblaciones recombinantes endogámicas (RI) (JI15 x JI399 y JI281 x JI399), localizando el alelo susceptible en el grupo de ligamiento V de *P. sativum*. La distancia entre estos dos marcadores fue difícil de estimar debido a que la frecuencia de recombinación de los dos cruzamientos es distinta pero están probablemente a una distancia de unos 20 cM. Los alelos *r* (semilla rugosa), *pa* (color de la hoja) o *det* (crecimiento determinado) pueden ser utilizados como marcadores clásicos para la introgresión de la resistencia.

De acuerdo a los resultados descritos, el modelo de herencia propuesto está basado en un gen principal recesivo presente en *P. abyssinicum* y en un grupo de genes modificadores, lo más probable dos, los cuales podrían estar presentes en *P. abyssinicum* y/o *P. sativum*.

Reacciones a la grasa bacteriana del guisante en distintos órganos de la planta

Resistencia a la grasa bacteriana del guisante normalmente ha sido testada mediante una técnica de inoculación del tallo asumiendo que las reacciones en las otras partes de la planta serían las mismas. Sin embargo, se observó que en las inoculaciones del

tallo muy próximas a su confluencia con la estípula, con la raza 3 en un cultivar con R3, algunas veces se manifestó una reacción de 'water-soaked' (susceptible) que se extendió en la estípula mientras que la reacción del tallo era la esperada necrosis (resistente). Se llevaron a cabo estudios más extensos para determinar cual era el alcance de las reacciones diferenciales a las siete razas de la grasa bacteriana del guisante en tallos, hojas y vainas. Los experimentos se realizaron en invernadero en una serie de accesiones de guisante (*P. sativum* y *P. abyssinicum*) con un solo gen de resistencia específica, con una combinación de genes de resistencia específica o con resitencia no-específica.

Una reacción resistente en el tallo no estuvo siempre asociada con resistencia en la hoja y/o la vaina. Esto se observó tanto para la resitencia específica como para la resistencia no-específica. Los genes de resistencia específica confirieron resistencia en el tallo constantemente, sin embargo, hubo variabilidad en las reacciones en la hoja y la vaina, las cuales dependieron de la interacción gen R y gen A (gen de avirulencia en el patógeno). El gen de resistencia específica R2 confirió generalmente resistencia en todas las partes de la planta a las razas 2, 5 y 7 (cada una de las cuales posee A2). Los genes de resistencia R3 y R4 individualmente no confirieron resistencia en la hoja y en la vaina en la mayoría de los casos, sin embargo, R3 en combinación con R4 tuvo un efecto aditivo en la resistencia. Las accessiones con resistencia no-específica fueron resistentes a las razas 2, 5 y 7 en todos los órganos de la planta y resistentes en el tallo pero con reacciones variables en la hoja y la vaina a las razas 1, 3, 4 y 6. Esto sugiere que la resistencia no-específica no siempre es expresada en la hoja y la vaina y que JI2202 y JI1640 podrían poseer R2.

También se estudiaron los efectos de las condiciones medio ambientales en la expresión de la resistencia, lo cual se llevó acabo en experimentos de campo en el otoño-invierno de 1998-99. Se utilizaron plantas crecidas en maceta del cultivar 'Kelvedon Wonder' y las accesiones JI2202 y JI1640 y se inocularon con las razas más importantes: 2, 4 y 6. La inoculación se realizó mediante pulverización de las suspensiones bacterianas intentando simular infección natural. Ocho semanas después de la inoculación todas las plantas *P. abyssinicum* estaban vivas y generalmente en un buen estado, aunque las plantas inoculadas con la raza 4 mostraron algún quebramiento de tallo. El daño por helada fue más severo en 'K. Wonder' que en *P. abyssinicum*, y como el daño por helada es un factor que según se ha publicado favorece la infección, entonces la tolerancia al frío de *P. abyssinicum* constituye un factor complementario para la reducción de la enfermedad en cultivos de siembra otoñal. La raza 4 fue la más agresiva de todas las razas tanto en experimentos de invernadero como de campo. La susceptibilidad de *P. abyssinicum* en la hoja a la raza 6 fue mucho menos obvia en campo. Esto sugiere que la combinación de la resistencia no-específica de *P. abyssinicum* con los genes de resistencia específica R3+R4 podría

ser una potente y eficaz combinación en condiciones otoñales/invernales.

POTENCIAL CONTROL BIOLOGICO BASADO EN LA ECOLOGIA DE LAS BACTERIAS ENDOFITAS

Detección de bacterias endófitas indígenas en el guisante

La incidencia natural de bacterias endófitas en el tallo del guisante y procedimientos para su aislamiento se estudiaron en un ensayo de campo en el sur de Holanda. Se desarrolló una técnica rápida de cribado para la detección de bacterias endófitas indígenas en el tallo del guisante que consistió en imprimir en el medio de cultivo 5% TSA ('Trypticase Soy Agar') secciones transversales del tallo que habían sido previamente desinfestadas con 1% cloro disponible y 0.1% Tween 80 durante 5 minutos. Once cultivares en el estado de floración fueron testados desde la base del tallo al ápice. La impresión de las secciones transversales proporcionó una estimación cuantitativa de los niveles de población. Colonización por bacterias endófitas fue detectada en cinco cultivares de los cuales Twiggy mostró la más alta y constante colonización y diferió significativamente del resto de los cultivares. El cultivar Twiggy se examinó en más detalle mediante el cribado de 20 plantas en el estado de vaina usando la técnica de la impresión desde la base del tallo al ápice. Además una sección del tercer y del cuarto entrenudo fueron desinfestadas, maceradas y sembradas en placa con un 'spiral plater' en tres medios de cultivo (5% TSA, R2A y SC). Las poblaciones endófitas decrecieron desde la base del tallo al ápice. Estas variaron de 10^3 a 10^7 CFU/g de tallo fresco, y comunmente fluctuaron en el rango 10^4-10^5 CFU/g. Las poblaciones difirieron significativamente en una de las parcelas de campo comparada con el resto. En una serie de 30 macerados de tallo, los conteos en el medio 5%TSA fueron un 17% significativamente más altos que en los medios R2A y SC. Las principales especies fueron *Pantoea agglomerans* y *Pseudomonas fluorescens*. *Ps. viridiflava* se detectó principalmente en uno de los bloques de campo y *Bacillus megaterium* exclusivamente en esa parcela. La eficiencia y la veracidad de la técnica de impresión fue comparada con el método tradicional de sembrado en placa. La relación entre ambos métodos mostró que la técnica de impresión es un buen método para la estimación aproximada de poblaciones en tallos de guisante en el rango 10^4-10^7 CFU/g.

Factores que influencian las poblaciones de bacterias endófitas en el guisante

El genotipo de la planta parece influenciar las poblaciones indígenas de bacterias endófitas en el guisante. Estudios más extensos fueron llevados a cabo sobre los efectos del tipo de cultivar combinado con estudios en la colonización por endófitos relacionada con el tipo de suelo, el estado de desarrollo de la planta y el órgano de la planta en tres ubicaciones en el centro-este de Holanda con diferentes propiedades del suelo (textura, contenido de materia orgánica y pH). Los genotipos de guisante estudiados fueron cuatro cultivares de *P. sativum* con una distintos destinos comerciales: Solara, Norli, 'Fortune' (línea parental en el programa de cruzamientos) y Twiggy (el cultivar que mostró el más alto nivel de colonización por endófitos); y una accesión de *P.abyssinicum*, JI2202 (línea parental en el programa de cruzamientos). En cada ubicación hubo dos campos con dos bloques por campo y cinco parcelas por bloque correspondiendo a un diseño completo randomizado de bloques. Las plantas fueron muestreadas en el estado de plántula y de floración. Secciones de raíces y tallos se procesaron en grupo (cinco plantas) o individualmente con la desinfección de la superficie del órgano de la planta y 'spiral plating' en el medio de cultivo 5% TSBA. La caracterización de las especies bacterianas se realizó mediante el sistema BIOLOG y el análisis de los ácidos grasos de membrana. Los conteos bacterianos fueron analizados usando los métodos 'restricted maximum likelihood' (Genstat 5) y estadistica Walds.

En el estado de plántula no se detectaron interacciones y se observaron diferencias significativas para tipos de suelo, genotipo y órgano (raiz-base de tallo). La más alta colonización en el estado de plántula para cada factor considerado independientemente fue el suelo ligeramente arcilloso, cv. Twiggy y la raiz. En la floración, las interacciones genotipo x órgano y ubicación x órgano fueron significativas. No hubo diferencias significativas entre ubicaciones o genotipos. El suelo ligeramente arcilloso representó la más alta colonización por endófitos en raiz y tallo. JI2202 fue el genotipo más colonizado en la floración. Poblaciones en raíces o en la base de los tallos fueron significativamente más altas que en la parte media del tallo. Se observó un aumento de poblaciones endófitas en el estado de floración (de log 5.14 CFU/g en plántulas a log 5.56 en plantas en floración).

Se utilizó un total de 570 aislados para la identificación de las especies bacterianas con el sistema BIOLOG y el análisis de ácidos grasos de membrana. Las Gram-negativas representaron el 90% de la población total, aunque un hubo un aumento de las Gram-positivas en la floración, especialmente en el suelo ligeramente arcilloso (del 3% al 20%). Las principales especies detectadas fueron *Pseudomonas* sp. (54% de la población total), *Pantoea agglomerans* (11%) y *Stenotrophomonas maltophilia* (8%). *Arthrobacter* sp. y *Curtobacterium* sp. fueron las principales Gram-positivas (40% y

17% respectivamente de la población Gram-positiva). *Bacillus* sp. fueron raramente detectados. En una cierta ubicación, algunas especies sólo fueron detectadas en cierto(s) cultivar(es). *Arthrobacter* sp. *Ps. viridiflava* y *Ps. syringae* pv. *syringae* fueron detectados principalmente en la floración y confinados al tallo. *Ps. corrugata* fue solo detectada en plántulas en las tres ubicaciones, mientras que *Agrobacterium* sp. y *Acinetobacter* sp. fueron detectadas sólo en la floración. El ratio entre el número de *Ps. fluorescens* en la raiz y en el tallo fue aproximadamente 1 para los suelos arenosos y arcillosos y 2 para el suelo ligeramente arcilloso.

Inducción de las células 'L-form' (sin pared celular) de *Ps. syr.* pv. *pisi*

Una eficiente producción de las células 'L-form' *in vitro* constituye un paso esencial para el uso potencial de la 'L-form' de *Ps. syr.* pv. *pisi* como agente de control biológico de la patogénica *Ps. syr.* pv. *pisi*. El primer procedimiento de inducción se basó en el método descrito por Paton e Innes (1991) para *Ps. syr.* pv. *phaseolicola*. Las suspensiones bacterianas se agitaron durante 16 horas en un medio de inducción líquido con penicilina (5000 unidades/ml, LIM+P), fueron transferidas a un medio sólido sin penicilina (SIM-P) y posteriormente se realizaron una serie de transferencias a medios sólidos con penicilina (SIM+P). La inducción de formas bacterianas sin pared celular pudo realizarse en LIM+P y en el medio sólido se pudo observar células de estructura granular y con grandes vacuolas. Sin embargo, hubo un escaso crecimiento en las siguientes transferencias y cierta reversión al tipo con pared celular. Para superar estas limitaciones, se introdujeron nuevos procedimientos en el protocolo, entre ellos un test de viabilidad. El periodo de tiempo óptimo para la inducción en LIM+P fue menor para *Ps. syr.* pv. *pisi* que para *Ps. syr.* pv. *phaseolicola*. La viabilidad de las 'L-forms' en SIM+P fue baja y disminuyó con el tiempo. El número de formas parentales (con pared celular) se incrementó en las consecutivas transferencias. En la evaluación de una serie de concentraciones de penicilina y ticarcilina con ácido clavulánico mediante el 'spiral gradient endpoint' se observó que la concentración final inhibitoria en SIM+P para *Ps. syr.* pv. *pisi* era cuatro veces menor que para *Ps. syr.* pv. *phaseolicola*. El uso de ticarcilina combinada con ácido clavulánico dio lugar a una inhibición del mecanismo de resistencia. El método recomendado es una inducción en LIM+P (5000 unidades/ml) durante 6 h, transferencia a SIM-P la cual parece ser necesaria para la adaptabilidad y consiguientes transferencias a SIM+Ticarcilina

CONCLUSIONES Y PERSPECTIVAS

Un programa de mejora para la resistencia a la grasa bacteriana del guisante debe considerar tres factores principales: (1) la distribución, frecuencia y agresividad de las razas existentes, (2) la expresión diferencial de la resistencia en distintos órganos de la planta y (3) los recursos genéticos del guisante. Los genes de resistencia específica son principalmente dominantes, fáciles de manejar y generalmente confieren una resistencia completa. En este estudio se ha demostrado que esto es cierto en el caso de la resistencia en el tallo, sin embargo, distintas reacciones han sido vistas en la hoja y/o la vaina en la expresión de la resistencia de los genes R3 o R4 individualmente. La presencia de R3+R4 tiene un efecto aditivo y confiere cierta resistencia en hojas y vainas. La resistencia no-específica presente en *P. abyssinicum* confiere una resistencia cuantitativa a todas las razas de *Ps. syr.* pv. *pisi*. Sin embargo, al igual que con la resistencia específica, hay un cambio en las reacciones en la hoja y/o la vaina, y la resistencia a las razas 1, 3, 4 y 6 no es conferida de una manera constante. En condiciones de campo, la susceptibilidad en la hoja a la raza 6 es relativamente baja y la susceptibilidad a la raza 4 en la hoja es moderada con cierto quebramiento de tallo. La raza 4 es muy agresiva, y tanto la resitencia específica como la no-específica no son totalmente efectivas contra esta raza. Es probable que *P. abyssinicum* también posea el gen de resistencia específico R2. Un beneficio adicional en el uso de *P. abyssinicum* es su tolerancia al frío, ya que el daño por helada es un factor que favorece la infección. Con los recursos genéticos disponibles actualmente, las posibilidades para alcanzar una resitencia duradera incluyen la combinación de la resistencia no-específica, la cual confiere resistencia en el tallo a todas las razas, y resistencia en la hoja a las razas 2, 5 y 7; y los genes de resistencia específica R3+R4 para la resistencia a las razas 1, 3 y 4 y su aparente intensificación de la resistencia en el tallo a la raza 6.

El presente estudio ha resultado en el desarrollo de: (1) F5s con resistencia no-específica procedentes de los cruzamientos entre dos cultivares de *P. sativum* y dos accesiones de *P. abyssinicum*, (2) descendencia de los cruzamientos de estas F5s con cultivares que poseen resistencia específica (los genotipos R1, R3, R2+R3, R2+R3+R4) y (3) marcadores moleculares para la resistencia no-específica y su ligamiento con los marcadores clásicos *r*, *pa* y *det*.

Para el desarrollo de un programa de control biológico con bacterias, es esencial conocer la biología de la bacteria introducida y su interacción con el huésped. La investigación del presente estudio se ha enfocado en bacterias endófitas, un grupo importante de bacterias asociadas con plantas. Se espera que representantes de este grupo tendrán mejores caracteres para una colonización estable del huésped que las bacterias epífitas las cuales se han usado tradicionalmente como agentes de control

biológico. En este estudio se han desarrollado técnicas de detección para las bacterias endófitas en guisante y se ha analizado su incidencia natural en campo. Los niveles de población estuvieron principalmente en el intervalo 10^4-10^5 CFU/g. La influencia del tipo de cultivar en los niveles de población fue demostrada en dos ensayos de campo en dos años. Los estudios para el estado de floración del cultivo fueron más complejos debido a la interacción de los factores estudiados. La raiz y la base del tallo fueron más colonizados que la parte superior del tallo y hubo un ligero aumento de las poblaciones con la maduración de la planta. El suelo ligeramente arcilloso con el contenido más bajo de materia orgánica generó las poblaciones de endófitos más altas.

La predominancia de *Pseudomonas* sp. como endófitas parece estar relacionada con la temperatura, con una preferencia por temperaturas templadas. Debido a la especificidad de las poblaciones de bacterias endófitas por el genotipo y el estado de desarrollo del huésped y por el tipo de suelo, es importante tener en cuenta que aislamientos con un efecto de control biológico en determinadas condiciones de cultivo podrían no ser igual de efectivos en otras condiciones. La especialización de las bacterias endófitas está relacionada no solo con el nicho que colonizan sino también con el punto de entrada. Aunque la raiz está considerada como la principal forma de acceso a partir de la cual pueden diseminarse en otros órganos de la planta, endófitas tales como *Pantoea agglomerans*, *Ps. viridiflava*, *Ps. syringae*, conocidas también como epífitas comunes de los órganos aéreos de la planta, también pueden penetrar directamente a través de lesiones y orificios naturales en hojas y tallos. Los datos obtenidos en el presente estudio sobre la colonización de diferentes genotipos de guisante ha mostrado que los niveles de colonización por endófitos se mantienen suficientemente altos desde el estado de plántula hasta la madurez. La gran biodiversidad de tipos de endófitos abre posibilidades para la selección de estirpes bacterianas para el control de *Ps. syr.* pv. *pisi* por antagonismo, competencia en el parénquima del tallo o por resistencia inducida.

El desarrollo de un programa integrado para el control sostenible de la grasa bacteriana del guisante incluiría la selección para la resistencia específica y resistencia no-específica, utilizando test estándard para los genes de resistencia específica y marcadores moleculares para la resistencia no específica junto con la selección de específicas bacterias endófitas las cuales colonizan con poblaciones superiores de 10^4 CFU/g en ensayos de campo con diferented tipos de suelo. Se espera que el uso combinado de estos sistemas mejore la duración de la resistencia considerablemente.

Author's curriculum vitae

Margarita Elvira Recuenco was born on 15 September 1968 in Madrid, Spain. In 1992 she obtained the Agricultural Engineer degree, specializing in 'Fitotecnia', at the Polytechnic University, Madrid. The final six months of these studies was concerned with a project on the design of a mushroom farm in Cuenca, Spain. In 1993 she worked for six months for the enterprise TRAGSATEC, Madrid, monitoring the olive tree fly (*Dacus oleae*) in Cáceres, part of an EC programme for the improvement of the olive oil quality. From January to September 1994 she was a trainee granted by the COMETT (EC) programme in the seed enterprise Nunhems Zaden, B.V. Haelen, the Netherlands, gaining experience on aspects of breeding of lettuce, spinach, carrot and radish. From October 1994 to April 1995 she worked at the Proefstation voor de Champignoncultuur (Mushroom Experimental Station), Horst, the Netherlands, on resistance to *Verticillium fungicola* in *Agaricus bisporus*. In 1995 she was awarded by INIA, Ministry of Science and Technology, Spain, with a five year fellowship for a PhD study to be conducted at Plant Research International, Wageningen, the Netherlands and Horticulture Research International, Wellesbourne, UK. During her PhD studies she was also a visiting scientist for two months in 1997 at Auburn University, Alabama, USA and for two months in 1999 at John Innes Centre, Norwich, UK.

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