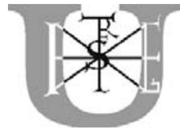

Szent István University
Faculty of Horticultural Sciences
Plant Pathology Department

NOVEL ANTAGONISTIC BACTERIA AS PROSPECTIVE AGENTS FOR THE BIOCONTROL OF SOME PLANT BACTERIAL DISEASES

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KHADIJA FARAJ AL-ARABI

Supervised by

Dr. HEVESI MÁRIA

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Denomination of doctral school: **Multidisciplinary Agricultural Sciences of Szent István University**

Division of Science: **Plant Production and Horticultural Sciences**

Head of school: **Prof. Dr. János Papp**
Doctor of the Hungarian Academy of Sciences
Head of Department of Fruit Sciences
Szent István University,
Faculty of Horticultural Sciences

Supervisor: **Maria Hevesi**
Candidate of the Hungarian Academy of Sciences
Szent István University,
Faculty of Horticultural Sciences,
Depatment of Fruit Sciences

.. **..**

Approval of Head of the school **Approval of the Supervisor**

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1. Introduction

Bacterial diseases of plants are usually very difficult to control and concern over potential toxicity of pesticides and over the continuing loss of appropriate, effective pesticides available for bacterial plant disease control have continued to increase since the 1970s. Most of the chemicals are used to control bacterial diseases of the foliage and of the aboveground parts of plants. Others are used to disinfect and to protect seeds, tubers, and bulbs or stored fruits and vegetables from infection. Some chemicals are used for soil treatment or disinfection. Chemicals applied on plants or plant organs can only protect them from subsequent infection and can not stop or cure a disease after it had started, some of these chemicals have local action, others have a therapeutic-eradicative-action and several are translocated systemically by the plants (Campbell, 1989, Agrios, 1997).

Bacteria multiplying inside the intercellular spaces of the tissues can hardly be reached with chemicals or antibiotics which have in use until now however, bacterial diseases can be controlled using preventive measures that are relatively effective and considered as cheap methods (Király et al.,1974). Earlier there was no incentive for the development or marketing of other systems and the problems that are considered important today, such as the concern about the environmental effects and safety of chemical pesticides were not fully recognized. Some of the early pesticides are potent toxins and have long-term effects on non-target organisms and are now prohibited in many countries, although they are in use in some other places where they still give cheap, effective control of some diseases, despite the health hazard and environmental damage they cause (Campbell,1989).

Modern pesticides have to pass very stringent tests for safety and for lack of any environmental hazard. However the fact remains that they are toxins and occasional examples of misuse, unexpected side effects, human and animal health hazard and appearance of new resistant strains do occur (Agrios,1997).

However a longer-term move by different interesting groups concerned about the environment, human, animal, and plant health proposes at least a reduction in the use of pesticides and more effective codes of practice or legislation to control their use. Unfortunately, it is reported that chemical control of bacterial diseases has been, generally, much less successful than that of fungal diseases. Therefore there is a need to find ways of controlling bacterial plant diseases other than chemicals and this has led to reexamination and improvement of many old practices and to the development of some new cultural practices for use in controlling bacterial plant diseases (Agrios,1997).

The use of biological control alongside limited chemical and cultural methods in integrated control programs is most successful and economical when all available pertinent information regarding the crop, its pathogens, the history of the disease, varietal plant resistance to diseases, the environmental conditions expected to prevail, land, labor, and costs are taken into account in developing the control programs (Gadoury et al.,1989).

Biological control of plant pathogens, that is, the total or partial destruction of the pathogen population by other organisms, occurs routinely in nature. There are several diseases where the pathogen can not develop in certain areas either because the soil, called suppressive soil, contains microorganisms antagonistic to the pathogen or because the plant that is being attacked by a pathogen has deployed antagonistic microorganisms by natural inoculation at infection court before or after the infection takes place (Cook, 1993).

Over more than 50 years biological control has advanced from a subject of basic research to a feasible component of an integrated disease management program by selection of effective antagonistic strains to control plant bacterial disease.

The first commercial use of biological control against plant pathogenic fungi was obtained in 1963 when *Heterobasidion annosus* the causal agent of pine root rots was controlled using spores of non pathogenic species of *Phlebia gigantea* which later became a commercially available product (Rishbeth,1963). Other commercial products were also marketed at this time such as *Trichoderma* spp., *Bacillus subtilis* strains, *Pseudomonas* spp. and now *Erwinia herbicola* strains which are at present the most widely used control agents for a number of diseases. During the last four decades efforts have been continued to improve biological control agents as commercial products for use in disease control (Riggle and Klos,1970 Baker and Cook,1974, Bruehl,1975, Cook and Baker,1983, Hornby,1990).

From the several bacterial diseases that received high attention from research scientists because of their serious effects on many national economical crops, we have selected the following:

Fire blight disease caused by *Erwinia amylovora* is one of these destructive diseases, that has caused great losses since first described in Hungary (Hevesi, 1996). It has been responsible for severe epidemics in apple and pear orchards (Németh, 1997, 1999). At present the disease threatens commercial fruit industries worldwide, it occurs on many fruit trees especially those that belong to the *Pomaceae* and *Rosaceae* families such as *Malus domestica*, *Pyrus communis*, *Cydonia oblonga* and *Cotoneaster spp*. It has spread in the central, southern, and eastern regions of the European continent and the USA (Steiner and Zeller,1996, Paulin,1997). In Hungary it had spread in last few years through many counties and during year 2000 it was in an epidemic distribution in fruit production orchards (Németh, 1999, Hevesi personal comminications).

Chemical control and cultural practices are not sufficient to control this disease or reduce the losses, so many research programs have been developed continuously to reduce the disease incidence or overcome the potential of the pathogen (van der Zwet and Beer, 1995). Several biological control agents were tested successfully against fire blight such as *Pseudomonas fluorescens, Erwinia herbicola,* avirulent strains of *Erwinia amylovora, Pseudomonas syringae,* and bacteriocins of species of *Enterobacteriaceae* (Jabrane et al.,1996, Vanneste,2000).

Other bacterial diseases that are considered important in Hungary Klement 1959 (cit. Ubrizsy,1965) are the bacterial spot diseases of *Capsicum annuum* and *Lycopersicon esculentum* caused by strains of *Xanthomonas vesicatoria* that cause destructive losses in these two economically important crops. Control measures are applied yearly but no complete eradication of the disease has been achieved so far. Biological control, however, provided good protection against the disease in small-scale plots. For example *Pseudomonas fluorescens* gave promising inhibitory effects (Colin et al.,1984, Tzeng et al.,1994).

Bacterial canker and wilt disease of tomato in Hungary Klement 1959 (cit. Ubrizsy,1965) is caused by *Clavibacter michiganensis* subsp. *michiganensis.* It has spread into different regions all over the world, causing considerable losses up to 70% of the yield mainly in out-door tomato crop production. In the last few years the disease has also been recorded in greenhouses (Shoemaker and Echandi,1976, Agrios,1997). Bacterial canker is one of the most difficult tomato diseases to control once it has established in vascular tissues of the crop for long periods and becomes seed-born, control measures used are not sufficient enough any more to eliminate the disease.

Biological control agents used against bacterial canker such as *Pseudomonas fluorescens, Bacillus* and S*treptomyces* species have an ability to inhibit the growth of *Clavibacter michiganensis* subsp. *michiganensis.* The role of inhibitory action of substances produced by these antimicrobial agents in reducing the disease severity and inhibiting growth of the pathogen has been demonstrated (Nishioka et al., 1997).

In Hungary, these diseases have been controlled by using different chemicals but these measures were insufficient enough to succeed. The biological control of soil-borne fungi such as *Fusarium*, *Rhizoctonia* and *Sclerotinia* species and some greenhouse pests, nematodes and weeds started only a few years ago by using different fungal antagonists such as a commercial preparation of the hyperparasite fungus *Coniothyrium minitans* called Koni against white mold caused by *Sclerotinia.* This preparation has been developed few years ago in Hungary (Vajna, 1987, Aponyi-Garamvölgyi,1989a,b).

Another example is the Hungarian *Trichoderma* biopreparation that has been tested for a total of nine years and found to be effective against almost all soil-borne pathogens (Aponyi-Garamvölgyi,1989a,b) also developed at Plant Protection Research Institute. A third Hungarian biopreparation in experimental stages based on *Ampelomyces quisqualis* an effective hyperparasite of powdery mildew has been developed by Vajna and Kiss (cit. Ilovai et al., 1996). Biological control of bacterial diseases using bacterial antagonists in Hungary had insufficient researches. Currently bacterial antagonists such as strains of *Pseudomonas fluorescens* are being tested (Rozsnyay et al., 1992, Dormanns-Simon et al., 1997, Biró et al.,1998)

Although biological control is not comprehensively practiced in fields all over the world as do other control measures and biological control of bacterial plant diseases with antagonistic bacteria is still insufficiently practiced, but this measure of disease control has been considered promising in initial research programs. The present study was conducted to identify biological control agents effective against the fire blight disease of *Pomaceous* and *Rosaceous* plants*,* leaf spot diseases of pepper and tomato and bacterial canker of tomato as a first study in Hungary with the following objectives:

- Isolation of antagonistic bacteria from the phylloplane
- Isolation of antagonistic bacteria from the rhizosphere (using different soil samples from Hungary and Libya)
- Application of *in vitro* methods for evaluation of effectivity of these antagonistic isolates
- Selection the most promising antagonistic isolates
- Estimation of the inhibition effects of these antagonists against plant pathogenic species of different genera for determination of antibacterial spectra
- Characterization of the selected antagonists by cultural, morphological and biochemical tests
- Development of *in vivo* methods to evaluate the effectivity of characterized antagonistic isolates by assessing disease reduction of fire blight of pomaceous plant species, bacterial spot of pepper and tomato and bacterial canker of tomato
- Comparing different methods for application of the antagonists using pre- and post-treatments of tomato and pepper plants with the pathogen under greenhouse conditions
- Persistence and establishment of the antagonists on foliage of apple trees for possible utilization of biocontrol agents in field conditions

2. Literature review

2.1. Control practices of phytopathogenic bacteria

Combination of control measures in an integrated strategy is required to combat a given bacterial disease successfully and economically (Gadoury et al.,1989). Infestation of crops with bacterial pathogens should be avoided by introducing and planting only **healthy seeds** or plants, **sanitation practices** aiming at reducing the inoculum in a field, adjusting certain **cultural practices**, such as fertilizing and watering so that the plants will not be extremely succulent during the period of infection in order to reduce disease incidence (Agrios,1997).

Crop rotation can be a very effective defense to phytopathogenic bacteria that have a limited host range. The use of crop varieties resistant to certain bacterial diseases is one of best ways of avoiding heavy losses if it is supplemented with proper cultural practices and/or chemical applications especially when environmental conditions favor the development of the disease (Agrios,1997).

Soil sterilization by steam or electric heat or solar radiation or by chemicals such as chloropicrin is possible in small areas only. **Seed-disinfection** can be achieved by using sodium hypochlorite or HCl solutions or by soaking the seeds for several days in a weak acetic acid solution. Foliar sprays of copper and zinc compounds, e.g. Zineb, Bordeaux mixture has given the best results. **Hot water treatment** is effective in some cases. **Solarization** of pear and apple trees to eradicate bacteria in fire blight cankered parts also effective (Katan,1981, Agrios,1997).

Several models for **disease monitoring** forecasting have been developed by different laboratories (Steiner and Lightner,1992, Berger et al.,1996, Billing,1996, 1999, in Europe; Smith,1996, 1998, 1999, in the USA). These models should help in assessing the proper time of application of control measures and the survey of the host plant, which may also contribute to the reduction of chemical control measures. Disease monitoring forecasting is based on temperature, humidity, and rainfall measurements during the blossom periods of infection by *Erwinia amylovora* (Zoller and Sisevich, 1979) and also for populations of *Pseudomonas syringae* pv. *tomato* (Jardine and Stephens,1987) and many other bacterial diseases (Caristi et al.,1986).

Chemicals have been used to control bacterial diseases for decades but they are generally much less successful than the chemical control of fungal diseases. The traditional bactericides used to control bacterial plant diseases such as foliar sprays are **copper compounds** such as

copper sulphate, copper oxychloride and copper hydroxide. These compounds are most frequently used for the control of bacterial leaf spots and blights. Zineb, Maneb, or Mancozeb mixed with copper compounds are also used Their effect is protective, which makes frequent sprays necessary (Bruehl,1975, Agrios,1997).

Copper may cause phytotoxic or contact effect such as rusting, discoloration and cracks, furthermore copper accumulation in the soil leads to a decline in plant vigor and inhibition of other microflora. Bacterial strains may became resistant to copper fungicides (van der Zwet and Beer,1995, Agrios,1997).

Antibiotics were used during the 1950s for crop protection against bacterial and fungal diseases and are still used in some regions while prohibited in others such as streptomycin sulphate formulations that are used in successful results in most countries (including Hungary against fire blight disease) and some of the results were encouraging when antibiotics were combined with other control measures. Streptomycin-oxytetracycline, kasugamycin and many other antibiotics can bind to bacterial ribosome and inhibit protein synthesis or kill the bacteria by acting as bactericides.

Streptomycin mixed with oxytetracycline is used in control of fire blight of pome fruits and could delay the appearance of resistant strains (Vanneste, 2000). Antibiotics are also used to control black rot of cabbage, bacterial spot of peach, tobacco wildfire, citrus canker, and some ornamental diseases, but not against *Agrobacterium tumefaciens* of grape because they cannot eradicate bacteria that survive systemically (Király, et al.,1974, Agrios,1997).

These antibiotics can be applied as sprays onto plant parts or dips for transplants. A few days after spraying, the antibiotic content in the plants gradually decreases, therefore, repeated spraying weekly is necessary. When spraying is neglected for longer than 10 days, a successful result cannot be insured (Király et al.,1974).

Antibiotics have not been effective enough in controlling certain bacterial plant diseases due to the following **problems**:

- Non-persistence or instability.
- Phytotoxic side effects.
- High costs
- Development of resistant bacterial populations.
- Gradually decreasing concentration of antibiotics in plant tissues a few days after first application may require several applications at short intervals (Rudolph,1989). Antibiotics had been used against certain bacterial diseases with different results, they are not effective for eradication, but some are only locally systemic. Therefore effective bactericides for crop protection against bacterial diseases are urgently needed (Jones et al., 1996).

2.1.1. Biological control

The subject of the present study requires the discussion of biological control in a separate chapter.

Biological control was defined by Baker and Cook as „the reduction of inoculum density or disease-producing activities involving growth, infectivity, aggressiveness, virulence and other qualities of a pathogen or parasite (fungus, bacterium, virus, viroid, prokaryote, nematode, and algae) in its active or dormant state by one or more organisms, accomplished naturally or through manipulation of the environment, host, or the antagonist, or by mass introduction of one or more antagonists" (Baker and Cook,1974).

Antagonism is actively expressed opposition and includes antibiosis, competition and parasitism also by stimulating the plant growth and induced resistance (Baker and Cook,1974, Blakeman and Fokkema,1982, Campbell,1989). Competition among microorganisms is mostly for food (carbohydrates, nitrogen and growth factors), for space (receptor sites on cells) or for oxygen. Antagonists may use more than one form of antagonism, the action of some antagonists may fit under more than one mechanism (Cook and Baker,1983, Campbell,1989, Cook,1993)

The importance of biological control considered as the latest environmentally friendly measure to control bacterial diseases of different crops. Biological control applies any means of controlling disease or reducing the amount or the effect of pathogens that relies on biological mechanisms or organisms other than man (Agrios,1997).

There are several diseases in which the pathogen cannot develop in certain areas either because of the suppressioness of soils where disease development on or in the susceptible host is suppressed, even though the pathogen is present in the soil or is introduced, or because the plant that has been attacked by a pathogen has also been naturally inoculated with antagonistic microorganisms before or after the pathogen attack (Weller,1988, Agrios,1997).

The antagonists may be an avirulent strains of the pathogen or other different organisms that destroy or inhibit the development of the pathogen by releasing into the soil substances toxic to the pathogen. Biological antagonisms, although subject to numerous ecological limitations, are expected to become an important part of the control measures employed against many diseases. Biological control works where chemical controls has been unacceptable or inadequate or unsuccessful as with crown gall of fruit trees by *Agrobacterium tumefaciens*, fire blight of pear and apple by *Erwinia amylovora* (Andrews,1992).

Biological control may be accomplished through cultural practices**:** habitat management to create an environment favorable to the antagonist, host plant resistance, or both, through plant breeding to improve resistance to the pathogen or suitability of the host plant to activities of antagonists, through the mass introduction of antagonists, nonpathogenic strains, or other beneficial organisms. The biological control of plant pathogenic bacteria is an alternative method to the application of chemicals, which may be accomplished through the destruction of existing inoculum, exclusion from the host, or the suppression or displacement of the pathogen after infection (Campbell,1989).

A biological control program involves three living systems; each may vary in itself, may interact with the environment and with the other two living systems. The physical relationship of plant pathogenic bacteria to its host during pathogenesis may be either as an epiphytic or endophytic so the more internal the pathogen during the host - pathogen interaction, the less vulnerable the pathogen to control by antagonists (Lindow, et al.,1978, Cook,1993).

2.1.2. Effectivity of antagonistic bacteria against pathogenic bacteria

For more than 20 years crown gall caused *by Agrobacterium tumefaciens* has been controlled biologically by dipping planting material in a cell suspension of *Agrobacterium radiobacter* strain K84.The effect is due to an antibiotic agrocin 84 (Kerr, 1980). This treatment results in a rather high level of disease control but the use of this strain had many difficulties. Later Jones and Kerr (1989) constructed a similar bacterial strain called K1026 which was more effective. Biocontrol efforts with *Erwinia amylovora* using *Erwinia herbicola* and recently *Pseudomonas fluorescens* and avirulent strains of *Pseudomonas syringae* and *E. amylovora* gained the highest attention from both a scientific and a practical point of view as effective biocontrol agent, (Riggle and Klos,1970, van der Zwet and Keil,1979, Vanneste, 2000). This subject is discussed in details below.

Pantoea. agglomerans the synonym of *Enterobacter agglomerans* and *Erwinia herbicola-Erwinia milletiae* is now used after application of numerical phenotypic analysis and DNA hybridization of its strains (Gavini et al.,1983,1989).

It has been isolated from many sources in addition to man, animals and insects, furthermore *Pantoea agglomerans* has been isolated from a long list of plants (Potrikus and Breznak,1977). There are many synonyms for the organisms grouped as *Pantoea agglomerans*, most of these names were given to strains isolated from various plant sources or soils. The first human isolates of certain biogroups of *Pantoea agglomerans* were reported in 1928 as opportunistic pathogens (Brenner,1983).

The bacterial cells of *Pantoea agglomerans* are straight rods, 0.5-1.0 x 1-3 µm, Gram negative, peritrichous flagellated, most strains produce yellow pigments and faculatatively anaerobic (Dye, 1969, Brenner,1983). The antagonistic effect of *P. agglomerans* was reported against many plant pathogens especially against fire blight disease on apple and pear and commercial products of *P*antoea *agglomerans* strains are now nearly available in USA, and are applied in small scale orchards as dry formulation against crown and root rot of apple trees in Canada (Brenner, 1984, Utkhede and Smith, 1997, Vanneste, 2000).

2.1.3. Products of biological control agents

Remarkable advances have been made in the sophistication of techniques used and in the number of specific antagonists studied in the last few years. New examples of successful biological control with resident antagonists continue to appear every year. Most of these antagonists are mass–produced, commercialized, and perhaps patented (Cook and Baker, 1983). In Hungary only a few products are available on the market to growers. Two such officially registered products are Mycostop (Kemira,FI) based on *Streptomyces griseoviridis* effective against soil-borne diseases caused by mainly *Fusarium* spp. and Trichodex WP (Makteshim-Agan,IL) based on *Trichoderma harzianum* effective against the gray mould disease also (Koni) based on Coniothyrium minitants against *Sclerotinia sclerotiorum* and *S. minor* (Vajna,1987, Aponyi-Garamvölgyi, 1989a, b, Dormanns-Simon,1994).

The commercially available products for biological control of plant pathogenic bacteria include only microbial products (containing living organisms) labeled as disease control agents (Baker and Cook;1974, Cook 1993, Commercial Biocontrol List, Anonymous;1999). The list of products are in the Appendix.

There are numerous bacterial plant diseases that occur in Hungary some can be controlled effectively others that difficult to control. Biological control programs are still not practically applied for these diseases, especially on some economically important crops that we have chosen for this study:

2.2. Fire blight disease

The name of the disease “fire blight” apparently was chosen because affected branches have persistent blackened leaves and the tree or shrubs appear as though scorched by fire.(van der Zwet and Beer,1995).

2.2.1. Importance and distribution of the disease

Fire blight caused by the bacterium *Erwinia amylovora*, is a very serious and most perplexing and quarantine disease of pome fruits. It is most destructive to pears and generally less to apple and quince and many ornamental plants in the families *Rosaceae* and *Pomaceae* which are also affected some quite severely (van der Zwet and Keil,1979, van der Zwet and Beer,1995). In recent years it has been described for the first time on Japanese plum (*Prunus salicina*) (Mohan and Thomson,1996) and Chinese mountain-ash (*Sorbus redliana*) (van der Zwet,1995). Its increased severity was also recorded on raspberry plants (Evans,1996) and lately it was reported to occure in *Fragaria* spp., *Spiraea* spp, *Populus* sp., *P. tremuloides*, and *Juglans* sp. (Paulin,1997).

Since the earliest observation of the disease in the Hudson valley of New York (USA) during 1780, the disease has been officially recorded in different countries all over the world (van der Zwet and Beer, 1995, Paulin, 1997). The disease has spread to New Zealand in 1938, to Canada in 1919 and South America in 1943 (Sobiczewski et al.,1997).

In 1957 the disease was introduced to the UK (Lelliott, 1968) and Egypt (Elhelaly et al., 1964, Abo-El-Dahab, 1984). The disease spread to 12 countries in Western Europe and 11 countries in the Eastern Mediterranean (van der Zwet and Beer,1995, van der Zwet, 1996). The isolation of *Erwinia amylovora* from pears in Japan was also confirmed (Sobiczewski et al.,1997).

In Hungary the disease was first reported by Hevesi (1996) in apple, later on pear (Németh,1997), by 1998 it was detected in 16 out of 19 counties where *Malus* and *Pyrus* orchards were attacked (Németh,1998, Steiner and Zeller,1996, Németh,1999). Based on the symptoms recorded in the orchards (2-years old cankered branches) the disease foci apparently present in the country before 1994 (Németh,1999). One of these apple orchards was at Nyárlõrinc in Bács-Kiskun county, (south-eastern part) where 43.5 hectares were destroyed after this epidemic and about 40 hectares of pear were destroyed by fire blight during the year 1997. Fire blight was found in Hungary in seven major host plants; *Malus sp., Pyrus sp., Cydonia oblonga, Mespilus germanica, Crataegus* sp., *Cotoneaster* sp., *Pyracanthae* sp, *Sorbus* sp. and *Chaenomeles* sp. (Németh,1999).

Fire blight attacks all aboveground organs of the host plant often leading to its death. Severity of the disease is a matter of its destructive character, ability of rapid dissemination and systemic distribution in the plant as well as the lack of effective control methods. The losses caused by fire blight in certain regions or even countries are often difficult to evaluate, particularly in a single year. Intensity and harmfulness of the disease vary from season to season and from region to region. In some years its activity increases very fast, but in others the disease is only of local importance. Both situations can occur in the same plants in the same field. Its appearance causes various implications, particularly in nursery production but sometimes also in the trade of apple and pear fruits (Garrett,1990, Hale et al,1996, Pauline,1997).

Losses in Hungary were considered as one of the most severe cases reported in the world, in terms of the number of different species infected and the broad area attacked. Its impact in the future is expected to be considerable and hard to estimate. In 1996, more than 60 000 trees were destroyed across the country. The infection level ranged from 4% to 60% of the trees (Németh,1999).

The spread of the disease is connected with the way of bacterial survival. According to van der Zwet (1994) there are four main forms in which *Erwinia amylovora* occurs: ooze, strands, epiphytic stage and endophytic stage. Mazzucchi (1994) point out that dissemination of fire blight on long distances can take place in three ways: transferring of nursery materials, bird migration and deposit of solid aerosols transported by high altitude air currents (van der Zwet,1994).

The disease spreads on short distances with the aid of insects, mainly on and in the body of honeybees, rain, wind, and pollens (DeWael et al., 1990, van der Zwet and Beer, 1995, Vanneste, 2000). *Erwinia amylovora* can also survive epiphytically on different organs of host plants. It was found on blossoms where it can multiply (Thomson, 1986) on leaves where it survives only for a short period (van der Zwet and Buskirk, 1984) and also its internal presence in the bud-wood can not be neglected (Bonn, 1979).

2.2.2. Characterization of the fire blight pathogen

Erwinia amylovora (Burrill) Winslow et al. (1920) the causal agent of the disease is a very important quarantine pathogen. It is a microorganism with only one form, the vegetative single cell, sometimes in pairs or chains. However, the bacterium is often found in a watery polysaccharide matrix, called ooze (Smith et al.,1988). Depending on weather conditions, it may take several forms, e.g. thread-like strands or liquid form. The pathogen has been found in low

numbers as an epiphyte on leaf and bud surfaces and as an endophyte in apparently healthy parenchyma tissues of the vascular system (Keil and van der Zwet,1972, Paulin,1997).

The bacterium produces numerous characteristic small, round, domed, mucoid, glistening colonies (van der Zwet and Beer, 1995). *Erwinia amylovora* strains are Gram-negative, rod-shaped (0.5-1.0 x 1.0-3.0µm), motile by peritrichous flagella, have a fermentative metabolism, and are oxidase negative and catalase positive (Sands, 1990, cit.Klement et al.,1990), it belongs to the family *Enterobacteriaceae* (Dye,1968,1981).

Different methods have been developed for identification of *Erwinia amylovora* including analysis of fatty acids (Sasser,1990, Wells et al.,1994), and serological methods (Hutschemackers and Verhoyen,1987, Gorris et al.,1996). Recently DNA-based methods gained large acceptance in the detection and identification of *E. amylovora* strains e.g. the polymerase chain reaction (PCR) (van Laere et al., 1985, Hale and Clark, 1990, Guilford et al,.1996).

Isolation of phages that lysing *E. amylovora* strains and their use for bacterium identification has been described by several authors (Baldwin and Goodman, 1963, Hendry et al.,1967). On the other hand Vanneste and Paulin (1990) had isolated 10 phages that lysing various bacteria from the *Erwinia* genus however, none of them appeared to be completely specific to *Erwinia amylovora*. *Erwinia amylovora* strains are highly homogenic and don’t have subgroups that vary in host range

The pathogen produces two pathogenesis-related substances that have been determined (Vanneste,1995). The first one is amylovorin, an acidic hetero-polysaccharide which is the main compound found in the cell coat and the bacterial ooze. It is not considered to be a toxin (Smith et al.,1990). The second one is harpin, a product of a gene cluster named *hrp* (Beer et al,1991). It is a protein with a molecular weight of 37 KDa, it is heat stable and glycine rich (Wei et al.,1992). Harpin causes a hypersensitive reaction on many hosts which suggests its involvement in plant resistance to many diseases.(Wei and Beer,1996).

2.2.3. The disease process

The development of fire blight disease closely follows the seasonal development of the host plant. Therefore, it is convenient to consider the life cycle of the disease as beginning in spring with the production of primary inoculum and the infection of blossoms, continuing through the summer with the infection of shoots or fruits, and ending in late summer or early fall with the development of cankers.

The primary infection occurs in spring when bacterial cells may be carried by wind, rain, or insects from holdover cankers. Furthermore resident bacteria present as epiphytic on the

surface or endophytic on or inside tree tissues may invade the blossoms or young shoots of the host plant where infection may restart as a secondary infections throughout the growing season (Lelliott and Stead,1987, van der Zwet and Beer,1995).

Secondary infections cause serious damage to the trees towards the end of the growing season. Cankers develop in the bark when progress of the infection slows and most bacteria die (van der Zwet, 1994, van der Zwet and Beer, 1995).

Inoculum may originate as bacterial ooze or strands produced on host organs. The bacteria can be disseminated by rain, wind, birds or humans by using contaminated pruning tools, and by insects, especially foraging honey bees (*Apis mellifera*) in those area where *Erwinia amylovora* exist in nectar or flower parts in ooze form (DeWael et al.,1990, Kiel and van der Zwet,1972, Vanneste, 2000).

Weather conditions greatly affect the development of fire blight disease. Multiplication of *Erwinia amylovora* occurs most rapidly between 24^0C and 29^0C. However, the pathogen can grow over a wider temperature range of 4^0C - 32^0C while disease development may occur at 13^0C - 25^0C under warm and moist conditions (van der Zwet and Beer, 1995). Rain is promotive in the development and dissemination of fire blight disease. The disease is more severe in regions under frequent rainfall periods occurs during early parts of the growing season followed by hot and humid weather. In addition, severe outbreaks of fire blight often follow hailstorms (van der Zwet and Keil, 1972, McManus and Jones, 1994b, van der Zwet and Beer, 1995).

2.2.4. Symptoms of the disease

Symptoms of fire blight are easily recognized with a few exceptions, they are readily distinguished from those of other pear and apple diseases. The most obvious symptoms on pear or apple are the scorched appearance of leaves on affected branches. When succulent shoots are affected, they bend characteristically to form the typical “shepherd‘s crook” (van der Zwet and Beer,1995).

Depending on the affected plant parts, fire blight may be called blossom blight, which occurs during spring in single flower or entire flower cluster. Affected plant tissues appear as water soaked then they wilt, shrivel, and turn brown to black as the infection progress. During warm, humid weather droplets of bacterial ooze often exude from peduncle. Other plant parts like

leaf and fruit spurs, succulent shoots and water sprouts or suckers are also very susceptible to infection (van der Zwet and Keil, 1979, Paulin, 1997).

Shoot or twig blight symptoms are similar to those found in blossoms, except that infection usually progresses more rapidly visible as dark brown to black in pear and light to dark brown in apple. In leaf blight leaves become infected with similar symptoms after bacteria enter stomata or wounds caused by insects, hail and wind whipping. Fruit, limb, and trunk blight are formed through infection of lenticels or wounds in the skin or from infected spurs. Infected pear fruit often show a dark-green, water soaked edge along the infected area, whereas apples exhibit a premature reddening of the area bordering the infection (van der Zwet and Beer, 1995, Paulin, 1997).

A sticky, milky to amber colored fluid or ooze often exudes from lenticels. In arid regions masses of bacterial strands have been observed on fruit, which later turn brown or black, shrivel and become mummified, as they remain attached to the spur. Limb and trunk blight or collar and rootstock blight are also found (canker extension). Symptoms on other hosts such as ornamental plants and nursery stocks are similar to those described for apple and pear (Lecomte, 1993, van der Zwet and Beer, 1995, Agrios, 1997).

Erwinia amylovora overwinters mainly in the margins of necrosis and cankers but sometimes is symptomless in the vicinity of these spots. In spring bacteria become active and multiply causing extension of the area of injury and leading to the appearance of the milky gray ooze on the surface of infected tissues. From hereon bacteria are disseminated by various agents and infect other plants through injuries (van der Zwet and Beer, 1995).

2.2.5. Control practices

Fire blight is rather difficult to control and control strategies need to combine different measures aiming to eliminate the source of the disease, reduce bacterial inoculum, limit its spread, prevent plant infection and reduce plant susceptibility. Early detection of infection foci is crucial, followed by estimation of possible crop losses and a choice of proper control measures (Paulin, 1997). The epiphytic and endophytic bacterial stages are important in long distance dissemination and may have significant consequences regarding quarantine regulations in countries without fire blight (van der Zwet and Buskirk, 1984, van der Zwet, 1994). Some countries such as Japan, Australia and South Africa closed their borders for fruit imports from countries where fire blight has been recorded. Also special quarantine procedures were elaborated in New Zealand (Hale et al., 1996).

Among **chemicals** copper compounds are recommended for the control of fire blight. Out of several formulations applied the most common are: copper hydroxide copper sulphate and lime (Bordeaux mixture) and copper oxychloride. In addition phosetyl aluminum had also some effects (Larue, and. Gaulliard, 1993, Saygili and Üstün,1996). Although the above mentioned compounds are quite good preventive bactericides they may cause rusting problems on leaves and fruits presumably due to weather conditions, mainly temperature (Vanneste, 2000).

Antibiotics have been also tested in various plants and different climatological/geographical regions. The first experiments on fire blight control with streptomycin were performed in the USA in the early fifties. But this antibiotic had registered in some countries only at the end of the decade, it has been widely used in apple and pear orchards. Streptomycin is considered as one of the most effective pesticides available for fire blight control (van der Zwet and Keil,1979, Psallidas et al., 1996, Agrios, 1997). All streptomycin-preparations are formulated as streptomycin sulphate (18%WP) at 100ppm/liter. Besides its preventive activity it is also locally systemic (van der Zwet and Beer, 1995). However, it was reported that streptomycin effectiveness diminishes rapidly in a few days after treatment (Vanneste, 1996).

During sixties in the USA the control exceeded even ten sprays per season, causing a development of streptomycin resistant population of *Erwinia amylovora.* Resistant strains were also detected in the early seventies (Jones et al,1996, Vanneste, 2000) Later, they were found in other regions of the USA, New Zealand and Greece (Thomson et al., 1993, Psallidas et al., 1996). Occasionally the resistance of bacteria to Streptomycin has been associated with chromosomal mutation (Schroth et al.,1979). Oxytetracycline is being used in those areas with streptomycin-resistant strains (Jones et al., 1996). However, it should be pointed out that streptomycin was superior to oxytetracycline in reducing the incidence of blight in blossoms inoculated with streptomycin-resistant strains (McManus and Jones, 1994a).

Kasugamycin appeared to be phytotoxic in apple and pear orchard trails causing rusting of flower petals, leaf damage and decreased fruitset, therefore it should be reserved for nurseries of some ornamentals (Aldwinckle and Norelli, 1990, Saygili and Üstün, 1996). Antibiotics, mainly streptomycin and oxytetracycline, are used in human and animal medicine and therefore they are not allowed to be applied for plant protection in many countries when improperly used. In Hungary, regulations permitted using some antibiotics such as kasugamycin (Kasumin 2L) and streptomycin sulfate in field sprayings with official permission for environmental and human safety. Presently in the USA, streptomycin preparations (Agrimycin, Agri-Strep) are used on an average of a few times per season, mainly at the time of blossoming and intensive shoot growth

(Sobiczewski et al.,1997). In some European countries streptomycin (Plantomycin, Fructocin) is usually recommended only for blossoming period (Deckers,1996).

Disinfecting of tools is an another important control practice because *Erwinia amylovora* can be disseminated by pruning tools. Potassium-manganous oxide (5%) and a quaternary ammonium compound at concentration of 10%, gave the best results as tool disinfectants (Nachtigall et al.,1986). Sodium hypochloride was also quite effective in eliminating bacteria from contaminated tools. Disinfection of cleaned pruning tools with methanol or ethanol solutions without flaming is not sufficient enough under orchard conditions (Deckers et al.,1987).

In England a 3-Phenolic-based disinfectant, or its substitutes were recommended (Billing,1983). Disinfection of tools used to remove infected parts of plants was executed with 4% Lysetol and 70% ethanol, 3% Sodium hypochloride and ethanol with flaming and hot water (70^0C). (Each solution was tested at different times following treatment). Tested compounds such as ethanol, hypochloride and hot water removed bacteria from tools after 20min (Hasler et al.,1996). Pruning of the infected plant parts should be applied against *Erwinia amylovora* infection during the appropriate time (either during dormant season or during summer in dry conditions) using always disinfected tools and considering the impact of weather conditions (Covey and Fisher, 1990).

Breeding programs for resistance to *Erwinia amylovora* in *Pyrus sp.* and *Malus sp.* as well as among other species of *Rosaceae* plants were conducted in many countries that were invaded with fire blight disease. In USA different breeding programs have been applied since the mid-19th century. The principle objectives of these programs were fire blight resistance and superior horticultural characteristics including late blooming, early maturity, fruit color and the production of high-quality, productive, late-keeping cultivars (Bell and van der Zwet, 1993).
Many well known cultivars were used to obtain thousands of seedlings for evaluation of resistant/sensitive characters; (van der Zwet and Keil, 1979, Aldwinckle et al.,1996, USA), (Fischer and Fischer,1996, Germany), (Hasler and Kellerhas,1995, Switzerland), (Hunter,1993, Canada), (Paulin et al.,1993, France) and (Bouma,1987, in Holland). Growing mainly the most resistant varieties of fruit trees and ornamental plants should keep nursery costs to a minimum (Sobiczewski et al.,1997).

Physical methods such as high temperature for control of fire blight disease, e.g. treatment of scion in hot bath (45^0C) for 3 hrs was sufficient to obtain total disinfection of fire blight pathogen. This method if accompanied by survival of buds, is very promising and could be useful in practice. (Keck et al.,1993, Sobiczewski et al.,1997). Solarization by increasing soil temperature through solarization of the whole infected tree in order to diminish losses caused by

removal of infected branches, could stop the development of cankers and eliminate the bacteria (Thomson, 1996).

Another new method for inhibition of *Erwinia amylovora* was developed by *in vitro* experiments using inhibiting potential of **plant extracts** from *Juglans regia, Berberis vulgaris, Rhus typhina*, *Viscum album* and *Hedera helix* applied as water suspension on agar media. It has been suggested that these plant extracts induce resistance to fire blight by stimulating some enzymatic activity leading to changes in pathogenesis related proteins e.g. β 1,3 glucanase and chitinase. 1% extracts from the apical meristem of Bartlett pear cultivar have demonstrated an *in vitro* bacteriostatic activity on *Erwinia amylovora* (Mosch et al.,1996)

Biological control. The first research programs have been developed more than 60 years ago in USA controlling the fire blight diseases (van der Zwet and Keil, 1979). Since then *Erwinia herbicola* and recently *Pseudomonas fluorescens* and avirulent strains of *Pseudomonas syringae* and *Erwinia amylovora* beside many others as potential biocontrol agents gained the highest attention from scientific and practical points of view (Vanneste, 2000).

The main problem with bacteria as biocontrol agents is their ability to survive on plant surface in natural conditions. It was proven that an *Erwinia herbicola* population colonizing apple flowers remained present throughout flowering and increased rapidly at petal fall (Goodman, 1965, Paulin, 1997). The population had increased 100 times at petal drop. *Pseudomonas*-populations predominated in some orchards at blossoming time. Interestingly about 30% of isolates collected in some orchards inhibited the development of *Erwinia amylovora* during *in vitro* conditions (Kearns and Hale, 1995). Kearns and Hale (1993) have also proved that a strain of *Erwinia herbicola* (Eh1087) applied to apple flowers were isolated after 4 days in 10-40% (as related to the initial population). After 10 days however, the population increased (400-800 times as compared to natural epiphytic populations of the bacterium). The most effective colonization by *Erwinia herbicola* took place when the flowers were treated at full blossom, resulting in 70-80% protection against *Erwinia amylovora* (Kearns and Hale, 1993).

Two antagonistic bacterial strains of *Erwinia herbicola* (Eh252) and *Pseudomonas fluorescens* (A506) with the pest biocontrol potential were applied separately and together, assuming that they could increase the protection of apple and pear flowers against *Erwinia amylovora* although, their mode of action are different. Although these bacteria are non-antagonistic to each other no synergistic effect either was observed between the two above mentioned strains (Vanneste and Yu, 1996).

The efficacy of *Erwinia herbicola* strain Eh381 was close to that of Streptomycin (Hickey et al., 1996). Zeller and Wolf (1996). found that different isolates originating from leaves and flowers of different host plants infected with fire blight were antagonistic to *Erwinia amylovora* under *in vitro* conditions and on pear fruitlets In field conditions, flowers of *Cotoneaster sp.* were sprayed preventively with various antagonistic bacterial species of the genera *Erwinia, Pseudomonas* and *Bacillus. Erwinia herbicola* strains were the most effective as compared to streptomycin (Zeller and Wolf, 1996).

Artificial media are suitable only to select organisms that produce metabolites in the medium and inhibit growth of *Erwinia amylovora in vitro* (Sobiczewski et al., 1997). About 150 isolates of *Pseudomonas spp.* were tested on agar medium to assess their antagonistic abilities towards *Erwinia amylovora* on the basis of antibiosis (Mitchell, 1993). Different researches have shown that species of *Pseudomonas* genus are good sources of beneficial chemical substances acting as bactericides (Wilson and Lindow, 1993). Further studies allowed separating seven chemical compounds from bacteria of the *Pseudomonas* genus, which inhibited the growth of *Erwinia amylovora* on agar medium. Tests conducted on pear fruitlets showed that only two of them inhibited effectively fire blight development during 5 days and non of them were phytotoxic (Mitchell et al., 1996).

In Belgium it was shown that 17 strains of *Enterobacteriaceae* produced bacteriocin, which appeared to be bactericidal to *Erwinia amylovora* (Thiry-Braipson et al.,1982, Jabrane et al.,1996). Antibiotic production by *Erwinia herbicola* is a very important mechanism of antagonistic activity and research has shown that this production is common (Beer and Rundle, 1980, Vanneste et al., 1992, Wodzinski and Paulin,1994, Vanneste,2000). Furthermore It has been emphasized that the protective action of *Erwinia herbicola* against *Erwinia amylovora* depends on its infection potential which is strongly related to weather conditions (Vanneste and Yu, 1990).

2.3. Bacterial spot disease of pepper and tomato

The bacterial spot or scab disease is seed-borne and probably occurs wherever tomato and pepper are grown extensively as field crops. The causal agent of the disease is *Xanthomonas campestris* pv. *vesicatoria* Doidge (1939) Dye(1978), which affect natural hosts like tomato (*Lycopersicon esculentum)* and pepper *(Capsicum annuum*), including ornamental pepper (*Solanum nigrum)* and the fruits of *Solanum tuberosum*.

2.3.1. Importance and distribution of the disease

The disease occurs worldwide, it causes losses in USA, Australia, Argentina, India, Sudan, Nigeria, Egypt, Italy, Russia, Austria, Romania, and Yugoslavia (Smith et al.,1988). It is an important disease of outdoor-growing crops causing considerable damage to the leaves and stems especially of seedlings, but it is most noticeable by its affect on the fruits. The disease is well developed in warm temperate climates ((Fahy and Persley, 1983, Lelliott and Stead,1987). In Hungary it was first described on tomato and pepper in 1959 by Klement (cit. Ubrizsy,1965) and later by Hevesi (1974), during last few years it has became worse and spread over different counties (Hevesi, 1993, Ledóné,1997).

2.3.2. Characterization of the leaf spot pathogen

The actual scientific name of the pathogen is *Xanthomonas vesicatoria* (Doidge) Vauterin et al. (1995) which is synonym. of *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye (1978). It is closely related to the species of the genus *Pseudomonas*. It is Gram negative, rod shape and belongs to the family *Pseudomonadaceae* (Bradbury,1984). As other *Xanthomonas* spp., their cells are 1.0-1.5 x 0.6-0.7µm in size with only one polar flagellum, straight or slightly curved, on the other hand, they never denitrifying nitrate. Colonies appear on the third day after cultivation, producing highly characteristic pale yellow lens-shape colonies on nutrient broth agar, or dark yellow pigments (Xanthomonadins) on YDC medium.

Xanthomonas species are plant pathogens, *Xanthomonas campestris* has many pathovars most of which are host specific. (Smith, et al., 1988). *Xanthomonas vesicatoria* was before a pathovar of *Xanthomonas campestris* (Elliott, 1951, Hayward and Waterston,1964a). Three biotypes can be distinguished. One type only infects pepper, another one infects tomato, the third type attacks both (Lovrekovich and Klement, 1965, Agrios, 1997). Strains originating from tomato and pepper behave differently on nutrient agar containing soluble starch. Pepper isolates do not hydrolyze starch, all tomato isolates strongly hydrolyze starch, except one group of isolates (Király et al.,1974).

It has been differentiated into four groups (races) (Cook and Stall, 1982) later, Ritchie and Dittapongpitch (1991) described ten races based on pathogenicity to *Capsicum annuum* cultivars. Also pathological, biochemical, serological and phage sensitivity tests have proved that *Xanthomonas vesicatoria* is not a uniform species.

2.3.3. The disease process

The pathogen overwintering as a seed contaminant in infected plant debris, in the soil and in other hosts. It can penetrate leaves through stomata and wounds and fruits through wounds. The disease spreads by rain, insects, wind, or direct contact of diseased plant parts. Infection of flower parts usually results in serious blossom drop. Optimum conditions for disease development are at a temperature of about 30^0C and relative humidity of about 90% (Smith et al.,1988). Numerous spots on infected leaves may cause defoliation or make the leaves appear ragged. Spots on leaves appear earlier and in greater numbers at 28-30^0C. Artificial inoculation of pepper may cause easily shading of the leaves (Király et al.,1974, Agrios,1997).

The pathogen penetrates the intercellular spaces through stomata. Multiplying bacteria cause blistering which in time results in the development of the bacteria through the cracks once again reach the surface, and from here splashing rain, wind and insects convey bacteria to healthy plants. Flowers and young fruits of pepper fall off together with the attached peduncles. A significant part of the damage occurs because pepper plants, which have lost their leaves, shed most of their flowers and therefore their yield is greatly reduced (Smith et al.,1988).

The disease causes significant damage on fruits where brown spots appear. Symptoms are quite obvious on green or red fruits. In green fruits, first tiny dark green and brown-black round bulging spots appear. Later they spread and coalesce due to the attacked and lacerated epidermis and cuticle. The developing fruit may crack, providing the opportunity for attack by secondary organisms. Such fruits may rot while still on the plant (Király et al.,1974, Agrios,1997).

2.3.4. Symptoms of the disease

In tomato often small, brown to black spots usually with chlorotic margins occur on underside of leaves. In stems these spots are round or elongated. Spots may coalesce causing cankerous stem lesions suberized with time. These symptoms eventually result in leaf blight and premature abscission. In fruits, spots appear as slightly-raised, corky scabs, usually irregular in shape, surrounded by water soaked margins (Fahy and Persley, 1983). Later in the season, spots become brown to dark, slightly sunken, with a rough, scab surface and the fruit epidermis rolled back. Spots that become irregularly circular with a yellow, translucent margin have brown to black, later parchment-like centers.

Spots may coalesce and form irregular streaks along veins or leaf margins. Edges and tips of leaves may become dead, dry and breakaway giving leaves a tattered appearance. Heavily

infected leaves turn yellow or brown and young leaves become distorted and die ((Király et al., 1974, Smith et al., 1988, Lelliott and Stead, 1987, Agrios, 1997).

In pepper the symptoms differ from those in tomato, mainly the leaves, peduncles and the fruits become infected. Small, irregular, elevated, water soaked, dark green and moist spots appear under the leaf surface, later these spots grow to 6 mm, margins turn dark brown or translucent with a whitish center. Spotted leaves turn increasingly yellow then fall off. Thus, strongly infected plants become defoliated.

Stem spots are oval-raised while in fruits spots have a pointed form with 1-2 mm in size a little raised and dark brown in color. Later 2-3 mm spots grow with a deeper center, darker color and broken margins, the epidermis becomes dark brown and develops a corky structure. Spots on the leaf surface may coalesce and form irregular streaks along veins or leaf margins. Edges and tips of leaves may become dead and dry and breakaway giving leaves a tattered appearance. Heavily infected leaves turn yellow or brown and young leaves become distorted and die. Small, brown or black raised dots or blisters form on the surface of fruits (Király et al.,1974, Smith et al.,1988).

2.3.5. Control practices

The effectivity of disease control measures depends on the use of bacteria-free seeds and seedlings, resistant varieties, crop rotations and sprays with fixed copper fungicides in the field. Under reasonably dry weather, premixed Bordeaux mixture and Zineb are also used (Agrios, 1997). Phosetyl Aluminum is considered to affect the pathogen indirectly and to induce natural resistance mechanism in treated ornamental plant species infected with bacterial spot and blight caused by *Xanthomonas campestris* (Chase, 1987). Seed treatments or dressings or hot water treatment (for tomato only), streptomycin spraying, and 3 -4 years' rotations were also recommended (Smith et al., 1988).

Biological control. The use of beneficial bacteria as biological control agents of bacterial spot diseases was reported during the last decade and gave promising results. Certain *Pseudomonas fluorescens* strains have been isolated that colonized tomato and sweet pepper seeds and showed an antagonistic activity to *Xanthomonas vesicatoria* (Campbell et al.,1998, Amat and Larrinaga,1992, Colin et al.,1984 and Tzeng et al.,1994) have shown that different strains of *Pseudomonas fluorescens* have clear inhibitory effects on *Xanthomonas vesicatoria* and many other *Xanthomonas campestris* pathovars under *in vitro* conditions. Protozoa have been also used against some pathovars of *Xanthomonas campestris* in soil and have promising results (Habte and Alxender,1975).

2.4. Bacterial canker and wilt of tomato

Bacterial canker and wilt of tomato (*Lycopersicon esculentum)* and also pepper (*Capsicum annuum*) is caused by *Clavibacter michiganensis* subsp. *michiganensis* Smith (1910) Davis et al.(1984). It occurs in different parts of the world and causes considerable losses. It is a major disease of out-door tomato as well as under greenhouse conditions and can be very difficult to control under protected cropping (Shoemaker and Echandi, 1976). The disease can easily cause yield losses of up to 70%. It is likely that in protected crops, where superficial symptoms can be difficult to diagnose without experience, losses are higher than realized (Smith et al., 1988).

2.4.1. Importance and distribution of the disease

The disease is widespread and destructive in the main tomato-growing areas, including the USA, Europe, Australia, New Zealand, Africa, Asia and south America. It was found to infect *Lycopersicon* and *Capsicum* species. *S*weet pepper *(Capsicum annuum)* as well as *Solanum mammosum*, *S. douglassi, S. nigrum* and *Nicotiana glutinosa* are also natural hosts. Furthermore a number of other solanaceous hosts are susceptible on artificial inoculation (Thyr et al., 1975, Fahy and Persely, 1983, Smith et al., 1988).

In Hungary the disease was first described in 1959 by Klement (cit. Ubrizsy,1965) and the causal agent was identified as *Clavibacter michiganensis* subsp. *michiganensis* in tomato plants grown in Hungary. It's importance in Hungary start to increase in the last few years (Hevesi, personal communications).

2.4.2. Characterization of the bacterial canker pathogen

According to Hayward and Waterston (1964b), Davis et al.,(1984) *Clavibacter michiganensis* subsp. *michiganensis* Smith (1910) Davis et al. (1984) is a Gram positive, slow-growing, non-motile member of the genus. Optimal growth is achieved in nutrient glucose agar where colonies are 1 mm in diameter after 3-5days and 2–3 mm after 7–8 days in culture. They are smooth, entire, convex, semi-fluid when freshly isolated but become butyrous with prolonged sub-culturing, with a color of pale yellow later becoming deeper yellow, opaque and glistening (Smith et al., 1988).

2.4.3. The disease process

The disease is known as bacterial canker or bird's eye spot of tomato. It is a typical vascular and parenchymatal disease on the stem with a wide range of symptoms well described in detail for greenhouse crops Smith et al., 1988) and for field crops by Strider (1969). Symptoms vary depending primarily on differences in cultural techniques used to grow the crop particularly nutrition, whether they are field or protected crops, the age of plants and the age at which they are affected and probably the infected cultivar as well. Young plants are more susceptible to wilting than older ones (Smith et al., 1988).

The vascular phase of the disease is probably less common than the foliar blight phase. A rapid collapse of infected plants occurs within a few days (Smith et al., 1988). The pathogen overwintering in or on seeds, in plant debris and in the soil. Spread of the infection occurs from seeds to cotyledons or leaves, but most infections result from penetration of bacteria through wounds of roots, stems, leaves, and fruits during transplanting, from windblown rain, and from cultural practices such as tying and suckering of tomatoes.

Once inside the plant, the bacteria enter the vascular system, move and multiply primarily in the xylem vessels, then spread into the phloem and cortex, where they form large cavities that result in the development of cankers (Lelliott and Stead, 1987, Agrios, 1997). The final, bird's-eye-like appearance of the spots, which have brownish centers and white halos around them is quite characteristic of the disease. In longitudinal sections of infected stems the vascular tissues show a brown discoloration, while large cavities are present in the pith and in the cortex and extend to the outer surface of the stem, where they form the cankers (Agrios, 1997).

2.4.4. Symptoms of the disease

Under protected cropping, vascular symptoms are usually not seen until plants reach or approach maturity. This is usually not before plants are 1.5–2 m high, symptoms appearing first on leaves in the region of the second or third truss above that being harvested. Leaves on lower parts of plants often have white, blister-like spots in the margins that become brown with age and may coalesce. On stems, shoots, and leaf stalks light-colored streaks appear, usually at the joints of petioles and stems (Smith et al., 1988, Agrios, 1997).

Later, cracks develop in the streaks and slimy masses of bacterial ooze come out through the cracks to the surface of the stem from which they are spread to leaves and fruits and cause secondary infections. Fruits develop small, shallow, water-soaked, white small spots, the centers of which later become slightly raised, tan to black colored with a rough cork-consistency. The

spots are framed by light green hallow. Sometimes fruits fail to develop and fall off or ripen unevenly. Infected seeds are often shriveled and black. The discoloration extends all the way to the fruits, both outward toward the surface and inward toward the seeds (Volcani et al., 1970, Lelliott and Stead, 1987, Fahy and Persley, 1983, Agrios, 1997)

In *Capsicum* spp. symptoms can be recognized as pale green, raised areas on leaves, which develop into irregular, corky pustules with a brown center. Pustules tend to collapse, leaving irregular brown spots. Leaf fall is a common consequence of the disease, however, bird's eye spots develop also in pepper fruits as well as in tomato. Vascular discoloration of infected pepper plants has not been observed in the field (Volcani et al., 1970, Fahy and Persely, 1983).

2.4.5. Control practices

Bacterial canker is one of the most difficult tomato diseases to control. Once it has been established in the crop, it can be extremely contagious. Detection of infected plants can be very difficult and there are no really effective means of chemical treatment. Furthermore, the pathogen can survive for long periods in the environment of the crop.

Copper-based sprays with three sprays at 10-days intervals should be applied followed by sprays at 3-5 week intervals (Fahy and Persely, 1983). Using proper sanitation such as removal of affected crop debris in protected crops is vital to prevent carryover of the pathogen to the next season (Agrios, 1997). Using pathogen-free seeds, application of streptomycin in the seedbed, and soil sterilization either by using chemicals, hot water, or high temperature is recommended, (Fahy and Persely, 1983, Smith et al., 1988, Agrios, 1997). Antibacterial activity of calixin as *in vitro* foliar spray has been shown to be effective against *Clavibacter michiganensis* and other Gram positive bacteria (Oros and Süle, 1980)

Biological control of bacterial canker disease in tomato has been indicated by Colin et al. (1984) who demonstrated that *Pseudomonas fluorescens* strains exhibit a various degree of antagonism towards *Clavibacter michiganensis* subsp. *michiganensis in vitro.* Other authors have also reported that species of *Pseudomonas, Bacillus,* and S*treptomyces* strains isolated from the rhizosphere of forest plants and soil have an ability to inhibit the growth of *Clavibacter michiganensis* subsp. *michiganensis* and other plant pathogenic bacteria and fungi. (Haansuu et al., 1998). Different reports have indicated the role of substances produced by different antimicrobial agents in reducing disease severity and inhibiting growth of the pathogen (Furuya et al.,1992, Gamard and El-Abyad et al.,1996, Nishioka et al.,1997, Okamoto et al.,1998).

Control of *Clavibacter michiganensis* subsp. *michiganensis* of tomatoes can be achieved by using *s*oil solarization, which demonstrate the thermal effect of solarization against the

survival of the pathogen in the soil. The rhizosphere isolates of *Bacillus, Pseudomonas,* and *Streptomyces* species are able to survive solarization and possibly contribute to the biological control of bacterial canker of tomato in solarized soils (Antoniou et al.,1995). In Hungary, a bacteriocin produced by Clavibacter *michiganensis* has been identified and demonstrated it's effect in case of some pathogenic strains of C. *michiganensis subsp. michiganensis* the causal agent of bacterial canker of tomato (Süle,1980).

The antagonistic effect of *Bacillus subtilis* on reducing the disease severity caused by *Clavibacter michiganensis* subsp. *michiganensis on* tomato was demonstrated, the effect of *Bacillus subtilis* on (Õrbottyán) soil has been also tested and found that the inhibition effect of the antibiotics produced by *Bacillus subtilis* can cause the formation of resistant colonies of the pathogen after few days (Ujfalussy, 2000). Applying *Bacillus subtilis* granules through water dropping to the cultivation can cause disease reduction of *Clavibacter michiganensis* subsp. *michiganensis* infection. It was proved that many antibiotics produced by *Bacillus subtilis* were wide spectra (Vanneste,2000) such as glycopeptide that has a role in plant growth stimulation (Bochow, 1992). *Bacillus subtilis* produce antibiotics that have an antagonistic effects on *Agrobacterium tumefaciens* (Utkhede and Smith,1993) and on different pathogens including *Xanthomonas vesicatoria* (Vas,1999). *Bacillus subtilis* also has better effect on fungal pathogens such as different species of *Fusarium oxysporum* (Bochow,1988,1989). *Bacillus subtilis* bacterial cells are Gram positive, 0.7 - 0.8 x 2.0 - 3.0 μm in size pretrichously flagellated forming endospores that are very resistant to adverse conditions (Vajna, 1987,Kerbs,1998).

3. Materials and Methods

3.1. Materials

Different types of bacterial and fungal species and strains also many plant species and cultivars were used in this study, beside some soil samples and antibiotics as following.

3.1.1. Pathogenic bacteria

For isolation of antagonistic bacteria the following bacterial species and strains were used from the collection of Szent István University, Faculty of Horticultural Sciences, Department of Plant Pathology (Table 1. and Table 2.).

Table 1. Plant pathogenic bacteria tested for selection of epiphytic and soil antagonistic bacterial isolates

Species	Code	Host/Place	References	Year
Agrobacterium vitis Ophel et Kerr (1990)	Av66	*Vitis vinifera* Hungary	Szegedi,E.	1992
Clavibacter michiganensis subsp. *michiganensis* (Smith) Davis et al. (1984)	Cm3	*Lycopersicon esculentum* Balmazújváros	Hevesi, M.	1997
Erwinia carotovora subsp. *carotovora* (Jones) Bergey et al. (1923)	Ec10	*Solanum tuberosum* Hungary		1972
Erwinia chrysanthemi pv. *chrysanthemi* Burkholder et al. (1953)	Ec533	*Phylodendron* sp. Hungary		1964
Pseudomonas savastanoi pv.*glycinea* (Coerper) Gardan et al. (1992)	PsR6	*Glycine soja* USA	Érsek,T.	1975
P. syringae pv. *mors-prunorum* (Wormald) Young et al. (1978)	PsmCs1	*Prunus avium* Budatétény	Hevesi, M.	1995
P. savastanoi pv. *phaseolicola* (Burkholder) Gardan et al. (1992)	PsPh 03	*Vicia faba* Tótkomlós		1994

Table 1. cont.

Table 1. cont.

Species	Code	Host/Place	References	Year
P. savastanoi pv. *savastanoi* (Smith) Gardan et al. (1992)	OLE1	*Oleander* sp. Hungary	Hevesi, M.	1998
Pseudomonas syringae pv. *syringae* van Hall (1902)	Ps26	*Prunus armeniaca* Budapest		1989
	Ps28			
	Ps29			
	PsV1			
	PsV2			
	PsDp1	*Citrullus lanatus* Medgyesegyháza		1997
P. syringae pv. *tomato* (Okabe) Young et al. (1978)	Pst6	*Lycopersicon esculentum* Fertõd	Hevesi, M.	1995
P. viridiflava (Burkholder) Dowson (1939)	Pv1	*Petroselinum crispum* Budapest		1998
Xanthomonas vesicatoria (Doidge) Vauterin et al. (1995)	Xv14	*Capsicum annuum* Szentes		1991
	Xv39			1992
	Xv60			1997
	Xv61			
	Xv62			
	Xv63			
	Xv64	*Capsicum annuum* Pécs		1998
	Xv65	*Capsicum annuum* Tordas		
	Xv66			
	Xv71	*Capsicum annuum* Kecskemét		
	Xv72			
	SO8	*Lycopersicon esculentum* Hungary	Klement, Z.	1964
X. hortorum pv. *pelargonii* (Brown) Vauterin et al. (1995)	Xhpel 136	*Pelargonium* sp. USA	Munnecke	1953
X. axonopodis pv. *phaseoli* (Smith) Vauterin et al. (1995)	Xap1	*Vicia faba* Ócsa	Hevesi, M.	1999
Fusarium oxysporum (Schl.) f. sp.*dianthi* (Prill. et Del.) Snyd. et Hans.	F. oxy. *Dianthi*	*Dianthus caryophyllus* Hungary	Folk, Gy.	1998
Fusarium oxysporum (Schl.) f. sp.*cyclaminis* Gerlach	*F. oxy. cyclaminis*	*Cyclamen persicum* Hungary		
Fusarium oxysporum Schl.	F. oxy.(-)	*Ocimum basalicum* Hungary	Nagy, G.	2000
Geotrichum candidum (Link.) Carmichael	Gc	Unknown Budapest	Human Health Services	1988

<u>Table 2.</u> Strains of *Erwinia amylovora* used for comparing their sensitivity to

antagonistic bacteria

Pathogen	Strain	Host/Place	References	Year
Erwinia amylovora (Burrill) Winslow et al. (1920)	Ea77	*Malus domestica* USA.	Goodman, R. N.	1958
	Ea898	*Pyrus communis* Pécs	Németh, J.	1997
	Ea910	*Cydonia oblonga* Pécs	Kovács, A.	
	Ea1	*Malus domestica* Nyárlõrinc	Hevesi, M.	1996
	Ea2			
	Ea3			
	Ea4	*Malus domestica* Sarkad		1997
	Ea6			
	Ea8			
	Ea9	*Pyrus communis* Sarkad		
	Ea10			
	Ea11	*Cotoneaster horizontalis* Sarkad		
	Ea12			
	Ea13	*Cotoneaster dammeri* Békéscsaba		
	Ea14			
	Ea15			
	Ea16	*Cotoneaster salicifolius* Pécs		
	Ea17	*Cydonia oblonga* Pomáz		
	Ea18			
	Ea19			
	Ea21	*Pyrus communis* Sarkad		
	Ea22	*Crategus* sp. Pécs		1998
	Ea23	*Pyrus communis* Zala megye		
	Ea24			
	Ea25			
	Ea26			

Table 2. cont.

Table 2. cont.

Pathogen	Strain	Host/Place	References	Year
Erwinia amylovora (Burrill) Winslow et al. (1920)	Ea28	*Crategus* sp. Zala megye	Hevesi, M	1998
	Ea29	*Cotoneaster horizontalis* Budapest		
	Ea30			
	Ea31	*Pyracantha* sp. Budapest		
	Ea33			
	Ea128	*Malus domestica* USA	Goodman, R. N.	1958

3.1.2. Plant materials

For isolation of antagonistic bacteria, young apple leaves and unripe fruits of cultivar. 'Starking' were collected from Budakeszi (Pest district) at May-June 1997.

3.1.3. Test plants

The following plant species were used:

Apple ***(Malus domestica)*** cultivars were used in different antagonistic tests:
'GRANNY SMITH', 'STARKING', 'LIBERTY', 'SPARTAN', 'PRIMA', 'CSANY1-M9', 'IDARED / M4', 'MUTSU / M9', 'IDARED / M9' and 'FREEDOM / MM106'.

Pear (*Pyrus communis*)

Quince (*Cydonia oblonga)*

***Cotoneaster* sp.** collected from different sites around Budapest.

Tomato (*Lycopersicon esculentum)* cultivar Kecskeméti 262

Pepper (*Capsicum annuum)* cultivar Cecei SH greenhouse seedlings.

3.1.4. Culture media

Nutrient agar, potato dextrose agar, King B, and different types of selective media were used, (media-details at Appendix).

3.1.5. Antibiotics

The following antibiotics from (HUMAN: INSTITUTE FOR SEROBACTERIOLOGICAL PRODUCTION AND RESEARCH, BUDAPEST) were used in the bioassay: penicillin, methicillin, ampicillin, streptomycin, neomycin, kanamycin, chloramphenicol, tetracycline, oxytetracycline, oleandomycin, erythromycin, polymyxinB, cephalothin, nystatin, and nitrofurantoin, trimethoprim and lincomycin (SIGMA CHEMICAL CO.USA.).

3.1.6. Soil samples

Samples were collected for isolation of antagonistic bacteria from different sites in Hungary (Fig. 1., Table 3.), and in northern agricultural parts of Libya (Fig. 2., Table 4.). But soil types and its pH were not determined specificaly.

Table 3. Soil samples collected from different sources in Hungary

Location	Code	Origin
Kecskemét	6	Tomato
Budapest	3	Plum trees
Budapest	5	Maple trees
Pécs	7	Grape
Tatabánya	8	Apple
Zsámbék	1	Pear

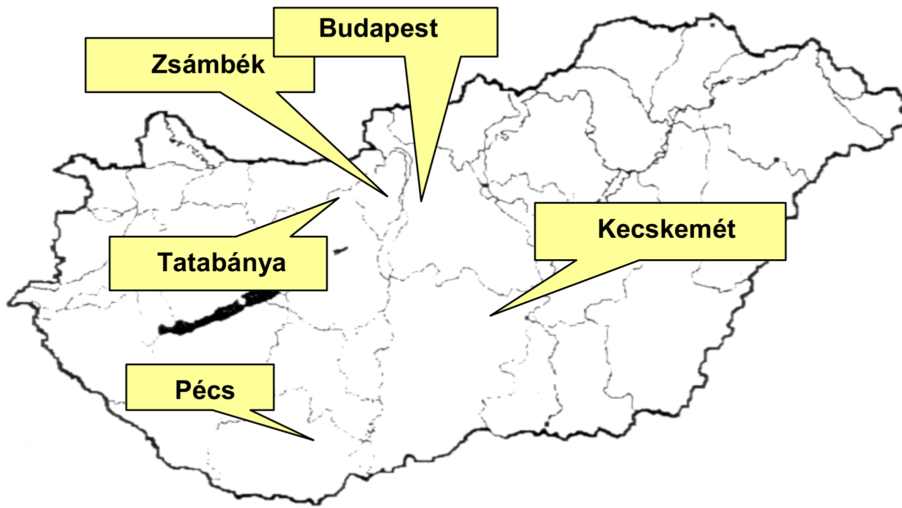


Fig.1. Map of Hungary illustrating sites of soil samples

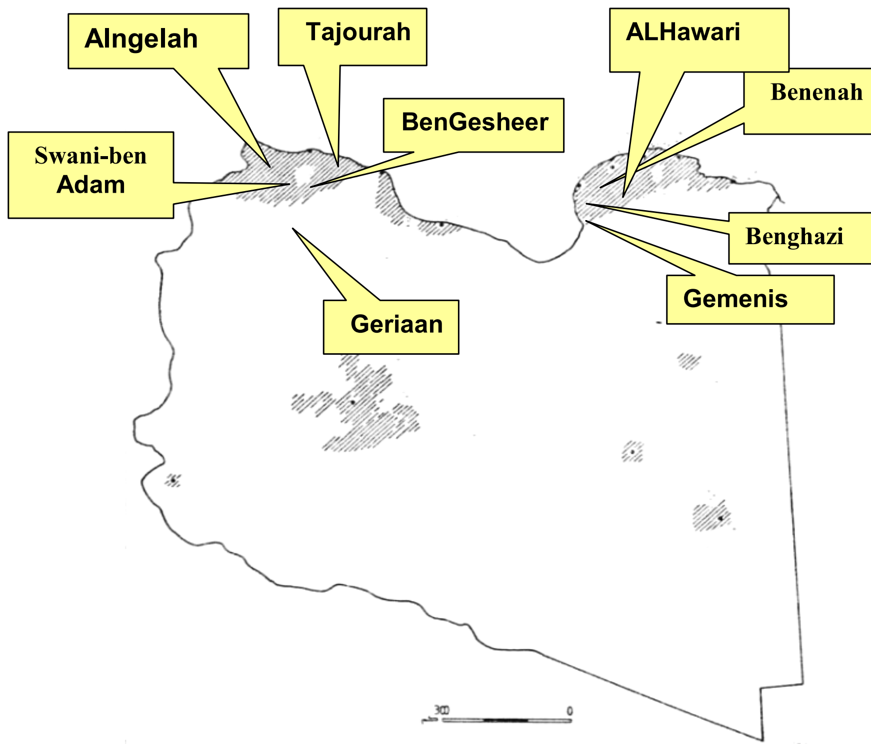


Fig.2. Map of Libya illustrating sites of soil samples

Table 4. Soil samples collected from different sources in Libya

Location	Codes	Origin
ALHawari	2	Olive trees
AlHawari	4	Almond trees
Alherah	9	Olive trees
Gemenis	10	Wild plants
Benenah	11	Figs
AlHawari	12	Olive trees
Tajourah	13	
Swani ben Adam	14	
Geriaan	15	
Gemenis	16	Figs
Tajourah	17	Olive trees
Benenah	18	Pome granites
Swani Ben Adam	19	Olive trees
Swani Ben Adam	20	Citrus trees
Alngelah	21	Olive trees
BenGesheer	22	
Benenah	23	Grapes
Benghazi	24	Olive trees
Benghazi	25	Citrus trees

3.2. Isolation and selection of antagonists from the phylloplane

Isolation of epiphytic antagonistic bacteria from apple trees cultivar 'Starking' was achieved from washing fluid of leaves and undeveloped fruits. Samples were diluted from ten fold to 10^{-3}. 100 µl of each dilution was plated on nutrient agar Petri dishes with a glass rod. Plates were incubated at 26ºC for 24-48hrs. Then the production of antibacterial metabolite of selected colonies was tested. **Selection** of pure colonies by transferring separated colonies onto Potato- dextrose agar (PDA), King's B (King, 1954), and Nutrient agar media-plates, followed by incubation at 26 ºC for 3- 5 days.

Streaking method was used. By streaked the indicator (pathogenic) strains at right angle to the tested antagonistic strain after three days incubation of the epiphytic isolate or at the same time (Fig. 3.).

The antagonistic effect was evaluated also by **double layer soft agar plate technique.** After 3 – 5 days incubation of the epiphytic isolate colonies were killed with chloroform vapor for20 min. then vaporizing the plates for half an hour. Then 3ml of 1% nutrient agar at 40ºC was mixed with 1ml of fresh bacterial suspension at dilution (10^{-7} -10^{-8} cells/ml^{-1}) and applied to each plate using different plant pathogenic bacterial strains (Table 1., Table 2. and Fig. 4.). Plates were incubated at 26ºC for 24hr or until inhibition zones have appeared around antagonistic colonies. Inhibition zones were measured in mm (DeVay et al.,1968, Weller et al.,1985, Psallidas et al.,1993).

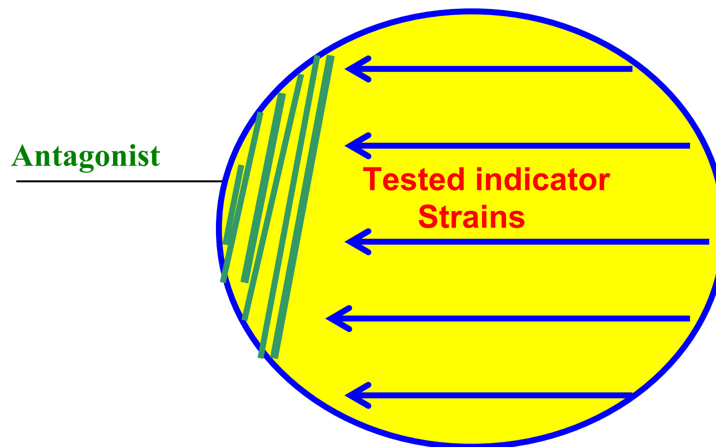


Fig. 3. Confirmation of antagonistic effect of the isolated bacteria by the streaking method

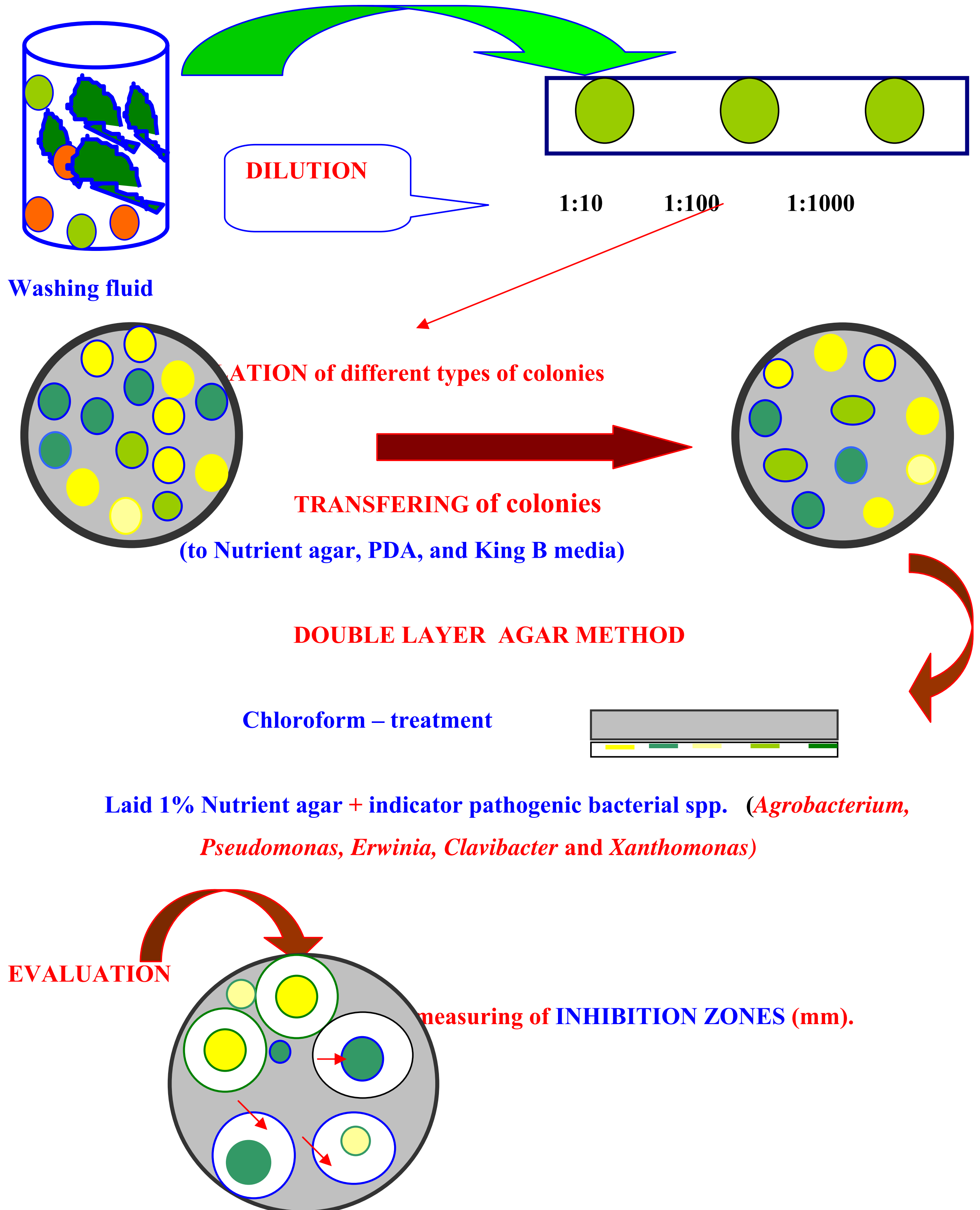


Fig. 4. Isolation and confirmation of antagonistic effect of the isolates by double layer method

3.3. Confirmation of metabolite production of selected epiphytic isolate against the pathogens

In order to determine whether the epiphytic antagonistic isolates have a real antagonistic effect so the inhibition zones due to the production of metabolites into the medium or that the inhibition zone is a result of pH changes of the medium by the candidate antagonist the following tests were carried out.

3.3.1. pH tolerance of the pathogens

We determined the tolerance of the pH of the test pathogenic strains of *Erwinia amylovora (*Ea1, Ea8, Ea17, Ea23), and *Xanthomonas vesicatoria* (Xv14, SO8). Strains were transferred to nutrient cultural media at different pH values: (4.5 – 7.0). The change of pH by the antagonistic isolate was also measured in potato dextrose broth (PDB) and Ayer's basal media after 7 days growth.

3.3.2. Effect of culture filtrate of epiphytic isolate by agar diffusion test

Antibacterial effects of culture filtrates prepared by selected epiphytic isolate were proved by different methods. Investigation of bacterial metabolites that have diffused into the medium by the epiphytic isolate was carried out. Potato dextrose broth (PDB) medium or basal medium (Ayer's et al.,1919) were used. The pH of the medium was adjusted to 7.0 before sterilization. The medium was inoculated with the respective antagonistic isolate (fresh culture) at ($5X10^8$ cfu/ml) followed by incubation at 26^oC for 6-7-days.

The suspension of the antagonist was centrifuged at 4000 rpm/20min. The supernatant was filtered through a 0.2 mm pore size membrane filter (NC 20- cellulose nitrate). Sterile culture filtrate (CF) was used without dilution and with dilutions at values: 1:50, 1:25, 1:12.5 and tested against the indicator pathogenic strains of *Erwinia amylovora, Xanthomonas vesicatoria* and *Clavibacter michiganensis* subsp. *michiganensis* that were grown in nutrient broth medium for 24 hours then plated by the double layer agar technique (Fig. 4. in section 3.2.1.).

After medium has solidified, four separated holes (11 mm.) were made by borer in every plate. 200 µl of each of the concentrated and diluted (1:50, 1:25 and 1:12.5) culture filtrate were poured into these holes. The last hole was filled with sterile distilled water as a control and the whole procedure was repeated twice for each species and each medium. The plates were kept in the refrigerator at 3^0C for 24 hours to promote diffusion of the culture filtrate. Petri dishes were

then transferred to an incubator at 26°C until inhibition zones around the holes appeared as a result of inhibition of growth of the pathogen demonstrating the antibacterial metabolites present in the culture filtrate secreted by the antagonist.(Staskawiczki and Panopoulos,1979, Klement et al.,1990, Zeller and Wolf,1996, Biró,1999).

3.3.3. Effect of culture filtrate of epiphytic isolate by poisoned agar plate

Minimal inhibitory concentration (MIC) of culture filtrate (CF) was determined by applying the following test: PDA medium was mixed before solidification with diluted culture filtrate (1:50, 1:25 and 1:12.5) adjusting uniformly to the final concentration. 100 µl of bacterial indicator strains of *Erwinia amylovora, Xanthomonas vesicatoria* and *Clavibacter michiganensis* subsp. *michiganensis* from broth (fresh cultures) at concentration of 10^{-3} cells / ml^{-1}. were sprayed onto these plates. Plates filled with the same quantity of sterile water and PDA medium served as controls. The treatments were incubated at 26°C for 2-3 days. The pathogenic bacterial colony forming units in each plate were counted (Klement et al.,1990).

3.4. Time dependent metabolite production of epiphytic isolate

The effect of different time intervals on the activity of antagonists was evaluated by growing antagonistic isolate on PDA medium plates every second day by streaking method. The antagonist and the indicator pathogen were cultivated together at the same time, then subsequently, the antagonist was cultivated after 2, 4, 6 and 8 days before transferring of the pathogen.

The test was applied by the double layer agar plate technique too. The antagonist was cultivated as mentioned above then was killed with chloroform vapor as in (Fig. 4.). The indicator pathogens were added in 1% melted agar and laid to the surface of plates of the antagonist. Resulting inhibition zones were measured in mm starting after 24-48hrs.

3.5. Persistence of epiphytic isolates in field conditions

In order to test the activity of antagonists and their efficacy after recovery from the spraying test, 24 hours old fresh cultures of antagonistic isolates were suspended in sterile distilled water (5x10^{8} cfu/ml) and sprayed on the original host plant apple trees, cv. ‘Starking’ two times per season in spring and summer. Samples of treated leaves were used to check the survival of bacterial population in selective medium.

Sampling of sprayed leaves was started at O-time by collecting random numbers of large and small leaves kept in a dry refrigerator until tested. The samples were put into sterile distilled water and shake in a rotary shaker for 10 minutes. Ten-fold dilutions of the washing fluid up to a dilution of 10^{-4} were made. 100µl of each dilution was plated onto Nutrient agar or PDA medium containing Trimethoprim as a selective antibiotic in order to inhibit the growth of microorganisms other than the tested antagonistic isolate where it was clarified by antibiotic assay that the epiphytic antagonistic isolate is resistant to Trimethoprim.

Samplings were continued weekly, three times per week and finished at the 4th week of each period. Colony numbers of the antagonistic bacterium were calculated and converted for colony number / cm^2 / leaf surface. The identity of the antagonistic bacterial population was confirmed by checking their antagonistic effect. Antagonistic effect was examined following the steps in (Fig. 4.) using double layer agar plate technique. The inhibition zones were evaluated and the respective isolate was compared to the original one (Kearns and Hale,1993).

3.6. Isolation and selection of antagonistic bacteria from the rhizosphere

Different soil samples originating from different places in Hungary and Libya were collected (Fig. 1., 2., and Table 3., 4.). 1g of each soil sample was suspended in 100 ml sterile distilled water and mixed in a rotary shaker for 20 minutes then left to precipitate for 5 hours. Ten fold serial dilutions up to10^{-5} were made. 100 µl of each dilution was plated onto Nutrient agar, PDA, and King B medium with a glass rod, then incubated at 26ºC for 2 - 3 days.

Selected approximately 450 colonies pure colonies were transferred to PDA plates, then incubated at 26ºC for 3- 5 days. Colonies were killed with chloroform vapor for 20 minutes then plates were left to evaporate for half an hour under sterile conditions. The double layer soft agar plate technique was applied as in (Fig.4.). Colonies which had inhibition zones were selected according to the magnitude of their inhibition effects. All positive antagonistic strains were preserved as shown below (Weller, 1988).

3.7. Sensitivity of bacterial pathogens, antagonistic isolates and soil microflora to antibiotics

For preparation of selective media bioassays on the effects of antibiotics on certain plant pathogenic strains had to be conducted. Different types of antibiotics in filter paper discs and solutions were used to test the bacterial pathogens, the isolated antagonists and the natural soil microflora as well. Discs containing different types of antibiotics were put at fixed spaces on the

surface of double agar layer plates with the tested strains of *Xanthomonas vesicatoria* and *Clavibacter michiganensis* subsp. *michiganensis* and soil suspension. In other tests, diffusion-method was used by made holes (11mm in diameter) in PDA plates and filled with an antibiotic suspension. Plates were incubated for 24-48 hr at 26ºC to record the inhibition zones around the discs or the holes. The scale used to measure inhibition zones was: Susceptible (S > 20mm), Moderately resistant (MR 11-20mm), Resistant (R < 11) (Ericsson and Sherris,1971, Leete,1977)

3.8. Preservation of antagonistic bacterial cultures

Cultures were maintained using different techniques. Preservation under **mineral oil** for long periods (from months to years); sterile paraffin oil was poured on the surface of nutrient agar tubes of fresh antagonistic isolates. The level of the oil should be well above the top of the agar and culture should be stored at 4°C in order to slow down the bacterial metabolism. Another technique involves preservation under **glycerol** (15 %) in vials and kept at -18C^{0} for 1-2 years (Lelliott, 1966, Sleesman, 1982). **Lyophilization** was carried out on these cultures for longer periods. The basic of this method is to rapidly freeze the bacterial suspension under high vacuum (10^{-3} - 10^{-4} Hgmm), (Gitaittis, 1987).

3.9. Confirmation the saprophytic ability of the antagonists

Applying the antagonistic isolates as biological control agents have to demonstrate that they couldn't cause disease symptoms on plants. The criteria for the fulfillment of host tests are excellent indicators of pathogenic or nonpathogenic properties of isolated antagonistic bacteria.

3.9.1. Plant inoculations

Tomato, pepper, and tobacco seedlings and apple leaves were inoculated by spraying with suspensions of epiphytic and soil antagonistic isolates at $5x10^{7}$ –$5x10^{8}$ cfu/ml. Saprophytic (or pathogenic) character of the isolates was evaluated by absence (or appearance) of symptoms or collapse or damages in tested plants.

3.9.2. Hypersensitive reaction (HR)

Tobacco plants (*Nicotiana tabacum*) were injected by different suspensions (10^6-10^7 -10^8 cells / ml) of selected epiphytic and soil antagonistic isolates. Intercellular spaces of intact, fully developed leaves were injected using a hypodermic needle. Plants were kept at room temperature.

Negative (or positive) hypersensitive reaction was evaluated by absence (or presence) of rapid collapse or necrotic symptoms of the leaves after 24-48-72 hrs. (Klement et al.,1964)

3.9.3. Soft rot test

The method involves washing and disinfecting fresh tubers of potato by dipping in 1-% sodium hypochlorite solution, cutting them into 7 - 8 mm slices. Holes were cut on the top of each slice and inoculated by several drops of a 10^8 cfu/ml fresh cell suspension of epiphytic and soil antagonistic isolates. The test was repeated with tuber slices inoculated with culture filtrate. Water-inoculated slices from each tuber were prepared for control, with several replicates for each tuber. All slices were placed into Petri dishes with sterile moistened filter paper then incubated for 24-72h at 26ºC. The inoculation site was poked with a sterile tooth-stick to detect presence of disintegrated tissues of the inoculated slices beyond the point of inoculation (Dickey and Kelman, 1988).

3.10. Cultural and morphological characterization of the antagonistic bacterial isolates

Colony shape, margins, elevation, surface appearance, opacity, texture, form and pigmentation of the antagonistic isolates on different media (nutrient agar, potato dextrose agar, Yeast dextrose chalk agar, King B medium) were evaluated following incubation at 26ºC for 3-days (see Appendix of media) (King et al.,1954, Lelliott and Stead,1987, Klement et al.,1990).

3.10.1. Presence of flagella

Using scanning- and transmission electron microscope for the presence (or absence) of flagella and their arrangements on antagonistic bacterial cells were determined of epiphytic and soil isolates by methods of MacNab,(1976). At Federal Office and Research Center for Agriculture, Wien, Austria and Central Laboratory of E. M. in SZIE University, Budapest-

Hungary.

3.10.2. Spore forming ability

Heating bacterial suspensions of epiphytic and soil antagonistic isolates in Nutrient broth to the point of sub-lethal effects, tends to age spores and enhances germination of endospores. Heat turbid treatment of bacterial suspensions in sterile tubes was conducted in a water bath at temperatures of 80ºC for 20, and 30 minutes. About 1ml of each heated suspension was pipette onto nutrient agar or PDA plates and incubated at 26ºC for 1-2 weeks.(Lelliott and Stead, 1987).

3.11. Biochemical characterization

For determination of the biochemical activity of antagonistic bacteria many biochemical tests were carried out for both epiphytic and soil isolates either by "traditional methods" or repeated by microset API E 20 (in case of the epiphytic antagonistic isolate only (see the Appendix)). The microset tests were chosen depending on the results of the traditional tests. In some cases microset tests were conducted together with the traditional biochemical tests (Klement et al., 1990).

The above-described tests for identification of epiphytic antagonistic isolates gave a basic confirmation for the family *Enterobacteriaceae* members. Additional confirmation by (API 20 E) tests was carried out in order to approve the identification to the genus and species level of the epiphytic isolate.

3.12. API 20 E test

The API 20 E microset is an easy and quick identification system for *Enterobacteriaceae* and other non-fastidious, Gram-negative, rod-shaped bacteria. It uses 23 standardized and miniaturized biochemical tests and a database. The most important biochemical characterizations which were originally devised for identification purposes were selected and used in combination with some other traditional biochemical methods as mentioned above (Klement et al.,1990)

The API 20 E strip consists of 20 micro-tubes containing dehydrated substrates (Table in the appendix). A fresh culture of the tested antagonistic isolate (at 10^9 cfu / ml), was homogenized and added to the test kit by micropipette (a few drops to each small tube) following, reconstitution the media. It was incubated at 37ºC for 24-72hours. During incubation, microbial metabolism produces color changes that are either spontaneous or revealed by the addition of reagents. The reactions are evaluated according to the Reading Table and identification is obtained by using the identification software API PLUS (Smith et al.,1972,

Holmes et al., 1978, Goor et al.,1984).

3.13. *In vitro* reduction of fire blight disease symptoms by antagonistic bacteria on different pomaceous plant parts

According to the literature when authors had determined the susceptibility or the resistance characters of pomaceous plants they observed that different plant parts reacted differently to the infection of *E. amylovora* (Sobiczewski et al.,1997). It gave for us the idea to evaluate the efficacy of the antagonistic bacterium on different plant organs.

3.13.1. Disease reduction on leaf discs

Sterile Petri dishes were filled with sterile polystyrol granules and sterile distilled water to act as a “moist chamber”. Suspension of epiphytic isolate 32
(at $6x10^{-8}$ cfu/ml) and *Erwinia amylovora* strain Ea1 (at $2x10^{-8}$ cfu/ml) were prepared. Fresh small and large leaves from different cultivars of apple were chosen and disinfected with 70% ethanol for few seconds.
Leaf discs were cut by a borer and immersed deeply into a suspension of the tested antagonistic bacterial isolate for 20 min as (pre-treatment) followed by immersion into a suspension of Ea1 as (post-treatment) for seconds. Discs were quickly picked up and dried under Laminar box for ½ hour, then treatments were repeated in reverse. Leaf discs were put onto polystyrol granules-filled plates, while additional discs were immersed in a suspension of *Erwinia amylovora* only as a positive control as shown in (Fig. 5.). Plates were kept at 26°C until symptoms appeared. The results were realized by two independent experiments and means of 4-replicates with 20-leaf discs in each replicate.

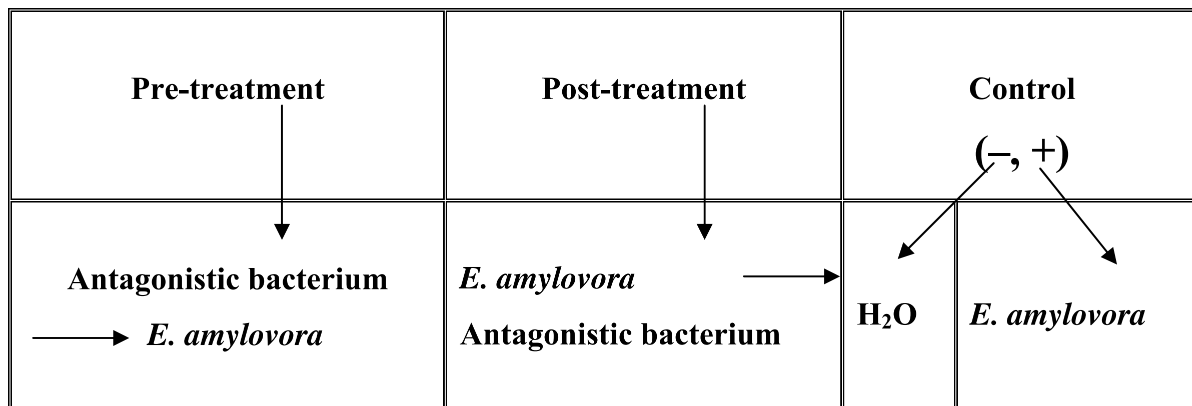


Fig. 5. Illustration scheme for different treatments in tests of antagonistic bacterial isolates

3.13.2. Disease reduction in complete leaves

Leaves from apple (*Malus domestica*), pear (*Pyrus communis*), quince *(Cydonia oblonga*), and cotoneaster (*Cotoneaster* sp.) were collected and disinfected with 70% ethanol then sampled in 6- replicates and three treatments as before.

Suspensions of antagonistic isolate (at $6x10^{-8}$ cfu/ml) and *Erwinia amylovora* strains (at $2x10^{-8}$ cfu/ml) were prepared. Moisturized 11- mm Petri dishes were filled with sterile glass mini balls or sterile filter paper. 6- leaves of each sample were immersed into a suspension of antagonistic bacterial isolate for 20 min (pre-treatment) then, for seconds into the suspension of *Erwinia amylovora (Ea*1) of apple, (*Ea*17) quince, (*Ea*23) pear, (*Ea*29) and cotoneaster. Reverse the treatments as above in (post-treatment). Leaf samples were picked up and dried under Laminar box for ½ hour, then dried and placed to moisturized conditions, while additional leaves were immersed in water as (- control) or a suspension of *Erwinia amylovora* as (+control). All plates were kept at $26^{o}C$ as shown in (Fig. 5.).Disease rating was calculated after 7-10 days by symptoms appearance.

3.13.3. Disease reduction in fruits

Antagonistic effects in some pomaceous fruits were evaluated by using the following bioassay: fresh, unripe young fruits were collected from trees of apple, pear and quince. Samples were disinfected with 70% ethanol for a few seconds, then left to dry. The suspension of the tested antagonistic isolate (at $6x10^{-8}$ cfu/ml) and the *E. amylovora* suspensions of the tested plant species (at $6x10^{-7}$ cfu/ml) were prepared.

Under sterile conditions fruits were cut into halves and a small wells (5mm. in diameter) on the intact surface was done and filled immediately with 60µl of bacterial suspension of the tested antagonist as (pretreatment) then, incubated under moist chamber for 24hr. after, filled with the pathogen *E. amylovora* suspension and reverse this treatment for (post-treatment) with 4–replicates for each one. Control treated either with water (-control) or with *E amylovora* suspension alone (+control) (Fig. 5.). All treated fruits were placed to a moist chamber and incubated at 26°C for 7days or until symptoms appear (Beer and Rundle,1983, Beer et al.,1984).

Antagonistic effects was tested also on flowers of cotoneaste*r* by grouping the flowers in beakers filled with water and sprayed with the epiphytic antagonistic isolate suspension ($6x10^{-8}$ cfu / ml) and a bacterial suspension *(*Ea 29) isolated from cotoneaster *(*at $6x10^{-7}$ cfu / ml)as pre-treatment and post-treatment, as applied before, Tightly closed the tanks with cellophane paper and incubated at 26°C for 24hours.

3.14. Quantitative analysis of pathogen survival in soil under influence of the antagonists

Studies of bacterial survival in soil were conducted in 4 independent experiments in order to study the efficacy of epiphytic and soil antagonistic isolates on recovery of *Clavibacter michiganensis* subsp. *michiganensis* the causal agent of bacterial canker of tomato, and *Xanthomonas vesicatoria,* the causal agent of leaf spot disease of tomato and pepper.

A fallow sandy soil **(**Őrbottyán, 0 - 30cm soil profile) received from the Experimental Station of the Institute of Soil Science and Agrochemistry of the Hungarian Academy of Sciences where it had been characterized (Table 5.). We determined its water absorbing capacity. A few grams of soil were dried at 120°C / 1hr. Droppings of water were added until the soil was saturated indicated by slowly passing through the filter paper, then the absorbing capacity of the soil was calculated by comparing the dry and wet weight and found as (30ml / 100 g^{-1} dry soil). The water capacity of the soil was considered in preparation of soil inoculations and watering.

Clavibacter michiganensis subsp. *michiganensis* (Cm3) and *Xanthomonas vesicatoria* (SO8) were grown in NA for 24hr. Each suspension at 10^{8} cfu / ml^{-1} was adjusted to the water capacity volume (30ml). For confirmation of antagonistic effects each ($100g^{-1}$) sterile dry soil sample at (80°C/ ½ hr) was mixed with ($2x10^{8}$ cfu/ml^{-1}) suspension of the above strains served as controls without antagonists. Meanwhile, treatments were prepared for combination of every antagonist and pathogenic strain calibrated to ($2x10^{8}$ cfu/ml^{-1}) in volumes of 15-15ml

respectively for each one. Soils were incubated at 26^0C and samples were taken for 5 weeks (Schaad and White, 1974)

Samples were taken continuously started at 0-time after few hours of treatments as follows: 1g of each sample was added to 100 ml of sterile distilled water and dissolved using a shaker for 5-minutes. After precipitation 100µl of each sample was diluted to 10^{-3}. Dilutions were plated to nutrient medium including antibiotics (according to pathogen tolerance. Selective medium with Nitrofurantoin for growth of *Xanthomonas vesicatoria* at 100ppm and with Lincomycin for growth of *Clavibacter michiganensis* subsp. *michiganensis* at 50 and 100 ppm. These antibiotics displayed effective inhibition on other microorganisms as well.

100µl of each dilution was laid on plates by an L- shape glass rod then all plates were dried and incubated at 26^oC until characteristics pathogenic colonies appeared. Numbers of colony forming units were counted and the antagonistic efficacy was evaluated comparing to control and in accordance with time.

Table 5. Characteristics of Őrbottyán soil type*

Characters**	Values
K_A (Plasticity index)	24 mg/100g soil
$CaCO_3$	7.2%
Humus	1.0%
Y1 (Hydrolyic acidity)	0.6 mg/100g soil
NO_3	18.0 ppm
NO_3+NO_2	0.5 ppm
P_2O_5	96 ppm
K_2O	114 ppm
Zn	4.8 ppm
Mg	32 ppm
water capacity of the soil	30-33%
pH in (H_2O)	7.5
Water capacity	30% (as determined in this study)

* Fallow sandy soil from (Experimental research field of the KISSAC)

**Determined in the Institute of Soil Science and Agrochemistry of the Hungarian Academy of Science

3.15. *In vivo* disease reduction by antagonistic bacteria

In our *in vitro* eperimental studies the efficacy of the epiphytic antagonistic isolate against different strains of *Xanthomonas vesicatoria* the causal agent of bacterial spots of tomato and pepper was demonstrated. There were few data in literature about the *in vivo* disease reduction tests using antagonistic bacteria. After the encouragable results we obtained against fire blight disease of pomaceous plants we start these experiments in greenhouse to demonstrate the efficacy of our epiphytic isolate against bacterial leaf spots on tomato and pepper.

3.15.1. Efficacy of antagonistic bacteria on disease reduction caused by *Xanthomonas vesicatoria* on tomato and pepper plants

Tomato cultivar 'Kecskeméti 262' and Pepper cultivar 'Cecei SH' seedlings were grown and transplanted under greenhouse conditions (at temperatures of: 26- 34°C and 85 - 95% relative humidity). Replicates of 10 tomato and 16 pepper plants were used for each treatment. One day before treatments all plants were covered with humidifying plastic bags.

Plants served as pre-treatment were first sprayed with a suspension of the tested antagonistic strain (at 5x10^{8}cfu/ml^{-1}). Others served as post treatments were sprayed with a suspension (at 5x10^{7}cfu/ml^{-1}) of *Xanthomonas vesicatoria* strains: of tomato (SO8) and pepper (Xv14*)*. After 24hours, repeating the treatments in reverse the order of treatments as described above. Another pot was served as a positive control with SO8 and *X*v14 respectively, negative control was with water. The pots were covered again for another 24hours, then, kept under the same conditions as described above.

Disease rating of tomato was evaluated according to the following degrees of scale (Fig. 6.).

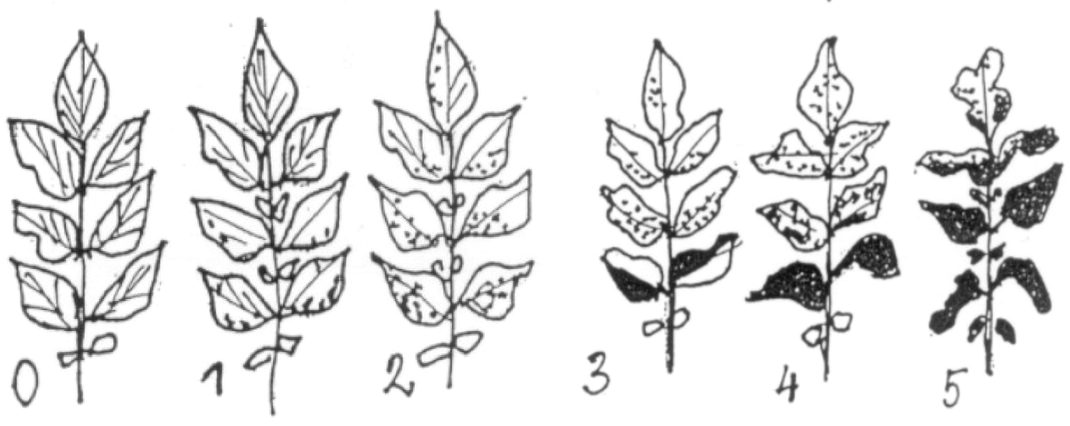


Fig. 6. Disease rating of tomato plants

Percentage of disease according to the necrotic spots/area:

0 no spots (disease symptom). 1 – 10
2 11 – 25 3 – 26 – 50
4 51 – 75 5 – > - 75 spots

Data were calculated according to the formula in disease rating. Pepper plants were also evaluated for the bacterial spot disease using the disease rating as described below.

3.15.2. Effect of antagonistic bacteria to disease reduction of bacterial spot caused by *Xanthomonas vesicatoria* and to development of pepper seedlings

Pepper seedlings cultivar 'Cecei SH' (4-5 weeks old) were arranged in pre-treatment and post-treatment, and control groups as described in (Fig. 6.) watered and covered with plastic bags for 24 hours. On the next day, fresh (24hours old) epiphytic bacterial suspension (at 5x 10^8 cfu/ml^{-1}) was prepared and sprayed on the pepper seedlings as pre-treatment, fresh culture of *Xanthomonas vesicatoria* strain Xv14 in suspension (at 5x10^7 cfu/ml^{-1}) was also prepared and sprayed on pepper seedlings as post-treatment and positive control sprayed with strain Xv14 only, then covered with plastic bags for 24 hours. On the next day, the treatments were reversed and the plants covered for another 24 hours. The following day, the cover was removed and the plants were watered every day and examined for symptoms appearance.

Disease symptoms were recorded 10-14 days after the beginning of the treatments by measuring the size of pepper leaves in mm^2 (leaf area) as a sign for plants development and counting numbers of fallen leaves starting at the end of the season. Evaluation of disease symptoms was conducted using the disease rating as mentioned below.

3.15.3. Length of persistence of the protective effect of antagonistic isolate against bacterial spot disease in pepper

5-6 weeks old seedlings of pepper cultivar 'Cecei SH' were watered and covered with plastic bags 24h before inoculation. Plants were first inoculated with a fresh 24hr culture of the epiphytic antagonistic isolate (at 5x10^8 cfu/ml^{-1}). All treatments considered as pretreatment. On the 1st day after treatment plants were sprayed with a fresh suspension (at 5x10^7 cfu/ml^{-1}) of

Xanthomonas vesicatoria strain Xv14, plants were covered with plastic bags for another 24h. The same procedure of pathogen inoculations was repeated on the 2nd, 3rd, 4th, 5th, 6th and 7th day-after the pretreatment as in (Fig. 7.). Treatments were followed by covering plants with bags for another 24h. Differences in temperature and humidity in the greenhouse during day and night or spring and summer that can affect multiplication of the bacteria were considered. Positive control was inoculated with strain Xv14 (at $5x10^7$ cfu/ml). All treated plants were watered every day and evaluated for the gradual appearance of bacterial spot symptoms. Evaluation of disease symptoms by counting the leaf spots was conducted by using the following scale of disease rating:

3.16. Disease rating

The disease rating for the evaluation of disease symptoms on treated plants was recorded by number of spots / leaf according to the following scale:

0 = no symptoms (spots)
1 = 1 – 10 (very few spots, difficult to record)
2 = 11 – 25 (spots appear clearly on most of leaves)
3 = 26 – 50 (necrosis starting in some leaves, infection in first lower leaves is starting)
4 = 51-74(strong necrosis, some leaves are dead)
5 = 75 – 100 (most leaves are partially or completely infected or dead with complete yellowing of leaves).

Results calculated according to the following formula:

$$Fi = \left(\frac{\Sigma\, a_i \times f_i}{n} \right)$$

Fi = Disease rating
ai = Disease categories.(0, 1, 2, 3, 4, 5)
fi = Number of infected plants per category
n =Total number of tested plants

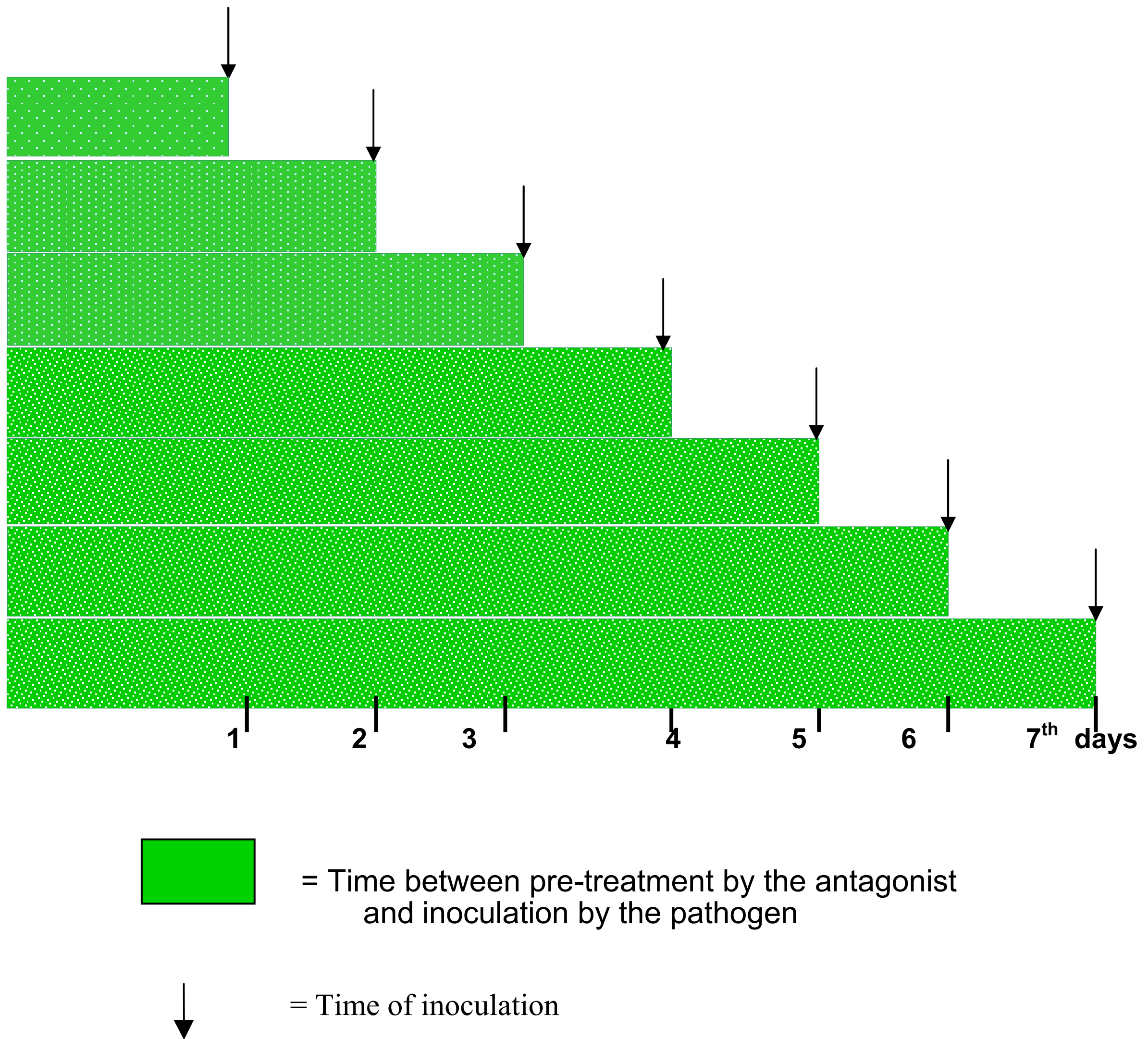


Fig. 7. Illustrative scheme of length of persistence of antagonistic isolate before inoculation by *Xanthomonas vesicatoria* strain Xv14 on pepper

4. Results

4.1. Isolation of antagonistic bacteria from the phylloplane

Screening for bacterial antagonists was carried out during the period of this study. The results indicated that among the phylloplane microflora of apple trees there are many antagonistic bacterial species that effectively could control phytopathogenic bacteria. Results showed that from a collection of 300 bacterial colonies tested for antagonism, numerous bacterial antagonistic isolates could be recognized (Table 6.).

Table 6. Antagonistic spectrum of epiphytic isolates that are effective towards phytopathogenic bacteria

Bacterial plant pathogens	Numbers of antagonists	Percentage of antagonists %
Agrobacterium vitis	10	4.7
Clavibacter michiganensis subsp. *michiganensis*	12	4.0
Pseudomonas syringae pv. *syringae*	28	9.3
Erwinia carotovora subsp. *carotovora*	16	5.3
Erwinia chrysanthemi pv. *chrysanthemi*	10	3.3
Xanthomonas vesicatoria	30	10.0
Erwinia amylovora	34	11.3

Results showed that the antagonistic spectrum of these bacterial isolates of apple foliage are effective against *Agrobacterium vitis* (the causal agent of crown gall and cankers of grapes), *Clavibacter michiganensis* subsp. *michiganensis* (the causal agent of bacterial canker of tomato), *Erwinia carotovora* subsp. *carotovora* (the causal agent of soft rot of vegetables *Erwinia chrysanthemi* pv. *chrysanthemi* (the causal agent of browning of stems on chrysanthemum), *Xanthomonas campestris* pv. *pelargonii* (the causal agent of spotting and wilting of pelargonium), *Xanthomonas campestris* pv. *phaseoli* (the causal agent of common blight of beans), *Xanthomonas vesicatoria* strains (the causal agent of bacterial spot of pepper and tomato), different pathovars of *Pseudomonas syringae*. (the causal agent of spots, blight and

canker diseases in different hosts) and different strains of *Erwinia amylovora* (the causal agent of fire blight of pomaceous plants).

4.2. *In vitro* effectivity of chosen antagonistic isolates

From different isolates of the apple phylloplane that had antagonistic effects on the basis of *in vitro* antagonistic tests against several species of the most important plant bacterial diseases, one isolate number (32) was selected according to its strong effects against most strains tested by its clear and strong inhibition zones and wide spectrum effects against numerous bacterial and fungal plant pathogens. The isolate (32) was chosen to continue the experimental work and was studied extensively by determine its taxonomic position and by application of more *in vitro* and *in vivo* tests and coded as Hungarian Isolate of Phylloplane (HIP32).

4.2.1. Preliminary antagonistic tests

Different antagonistic tests of the selected antagonistic isolate HIP32 were carried out using different strains of the main plant diseases under studying beside other indicator pathogens. The streaking method and the double layer agar plate technique were used after cultivated epiphytic isolate for 3 days The antagonistic effect was evaluated by measuring inhibition zones around the indicator strain in mm. An inhibition zone of more than 25mm was considered as strong effect while of less than 15 mm was considered as a weak effect. Results indicated that there were differences in sensitivity of different tested bacterial species to metabolites produced by isolate HIP32 according to the distances of inhibition zones (Fig. 8., 9. and 10.).

4.2.2. Testing epiphytic isolate HIP32 against fungal pathogens

Fungal species of *Fusarium oxysporum* and *Geotrichum candidum* were used *in vitro* spectra tests against antagonistic isolate HIP32 by growing the antagonist for 3-5 days before applying the fungal growth opposite to the antagonist. Results after 3 - 7days incubation indicated that isolate HIP32 had some effects on fungal pathogens as well as its clear effect on most of plant pathogenic bacterial species tested (Table 7. and Fig. 11.,12.).

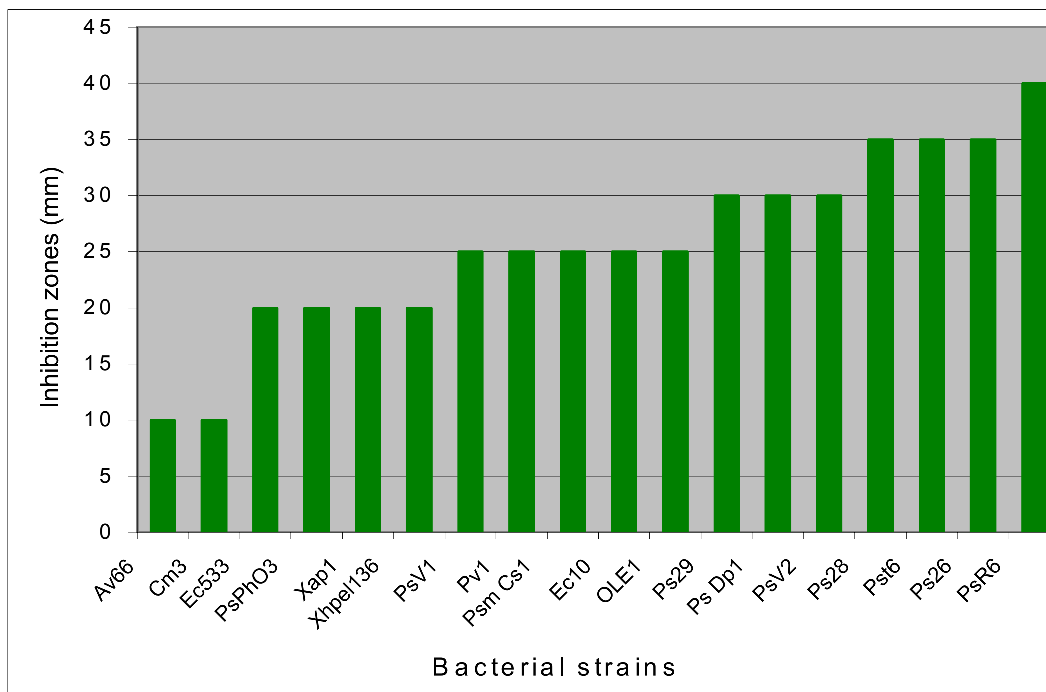


Fig. 8. Spectrum effects of isolate HIP32 towards different plant pathogenic bacterial strains

Table 7. Spectra of antagonistic effects of isolate HIP32 **towards different fungi**

Fungal pathogens	**Inhibition zones / mm**
Fusarium oxysporum (-)	20
Fusarium oxysporum f. sp. *cyclaminis*	25
Geotrichum candidum	25
Fusarium oxysporum f. sp. *dianthi*	35

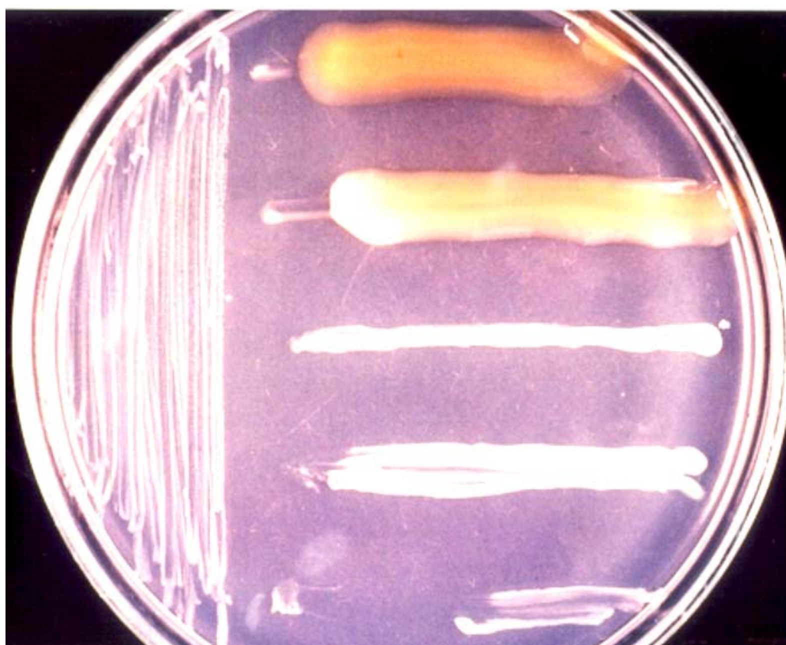


Fig. 9. Antagonistic effect of epiphytic isolate HIP32 **against from left: *Erwinia amylovora, Erwinia carotovora* subsp. *carotovora, Pseudomonas syringae* pv. *syringae* and *Xanthomonas vesicatoria* strains Xv14 and SO8 by streaking method**

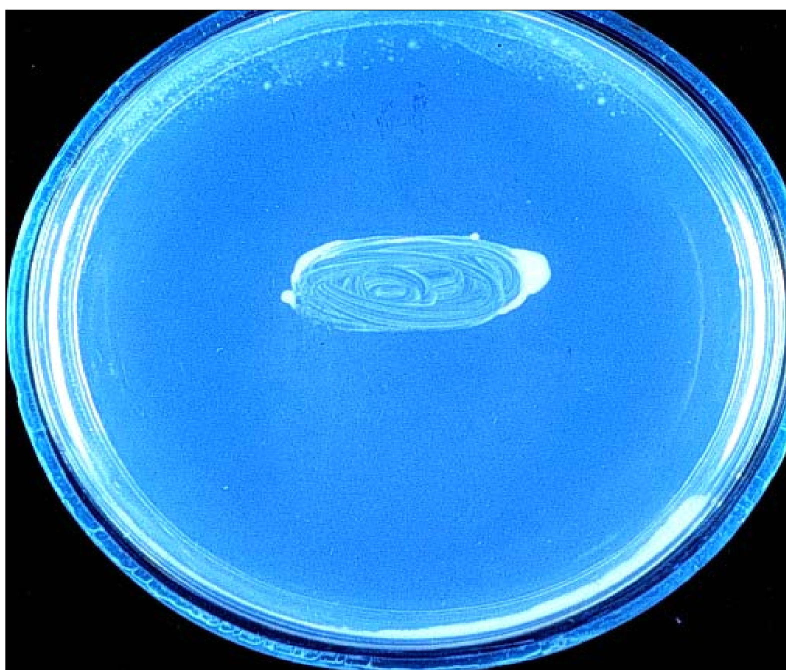


Fig. 10. Antagonistic effect of epiphytic isolate HIP32 **(in the center) against *Pseudomonas syringae* pv. *syringae* (around the plate) using double layer method**

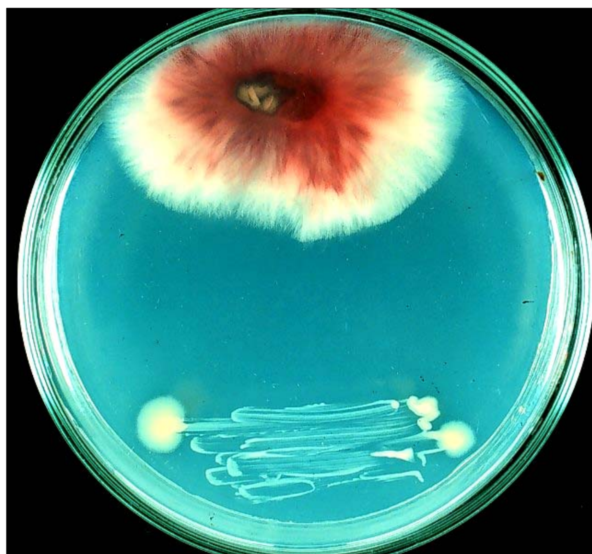


Fig. 11. Antagonistic effect of epiphytic isolate HIP32 **A= (dawn) against F*usarium oxysporum* f. sp. *dianthi* (up*):* B= *C*ontrol growth was more than 50%**

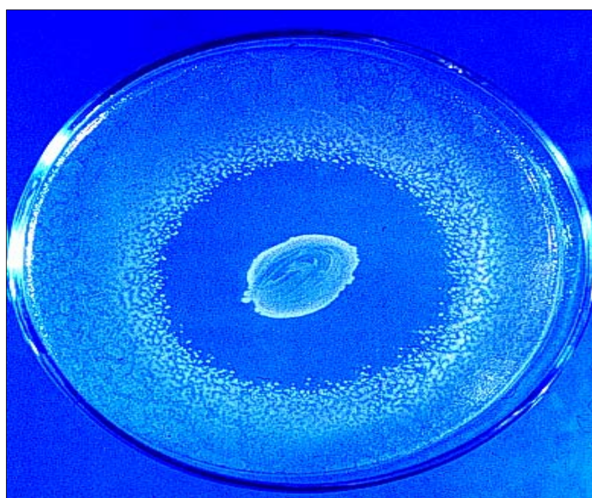


Fig. 12. Antagonistic effect of epiphytic isolate HIP32 **(in the center) against *Geotrichum candidum* (around the plate*)***

Results also indicated a wide spectrum and clear effect of isolate HIP32 on most strains of *Erwinia amylovora* and *Xanthomonas vesicatoria* the causal agents of the most important bacterial diseases chosen for this study. These pathogenic species demonstrated differences in their sensitivity to the antagonistic effects of isolate HIP32. In comparing the size of inhibition zones there were small differences in the sensitivity of these strains to isolate HIP32.

Strains Ea15 originated from *Cotoneaster dammeri* of Békéscsaba and Ea26 of *Pyrus communis* of Zala megye were less sensitive when compared to strains Ea1 and Ea8. Strain Xv63 of *Xanthomonas vesicatoria* originated from pepper of Szentes was also less sensitive than strains Xv14 and XV61 which were more sensitivite but no resistant strains were found. These results were considered in continuing the experimental tests with isolate HIP32 (Fig. 13a,b. and Fig. 14.).

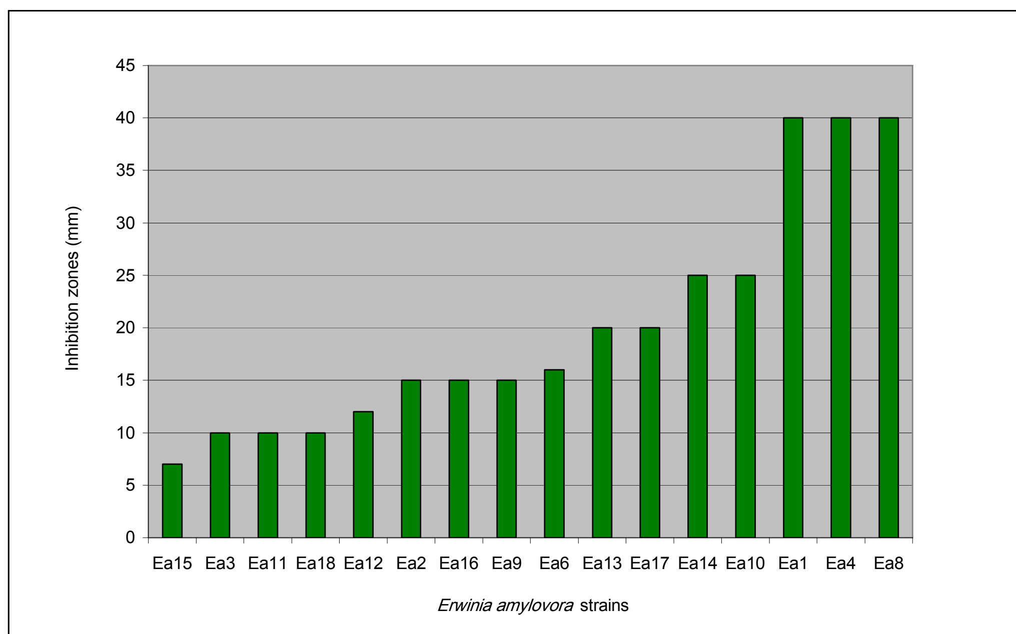


Fig.13a. Antagonistic effects of isolate HIP32 **towards different strains of *Erwinia amylovora***

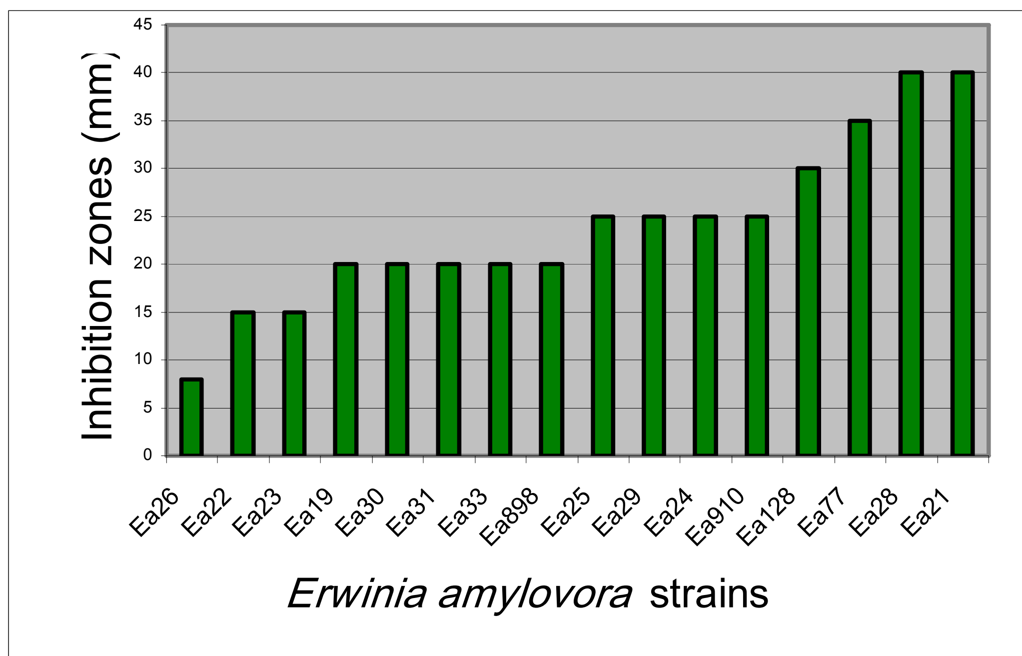


Fig.13b. Antagonistic effects of isolate HIP32 **towards different strains of** ***Erwinia amylovora***

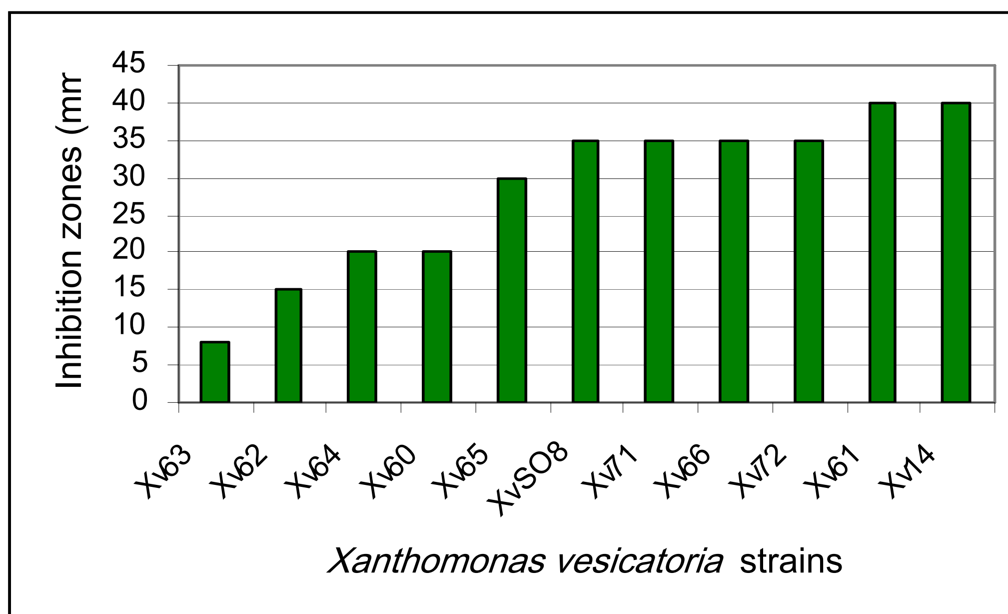


Fig. 14. Antagonistic effects of isolate HIP32 against different strains of *Xanthomonas vesicatoria*

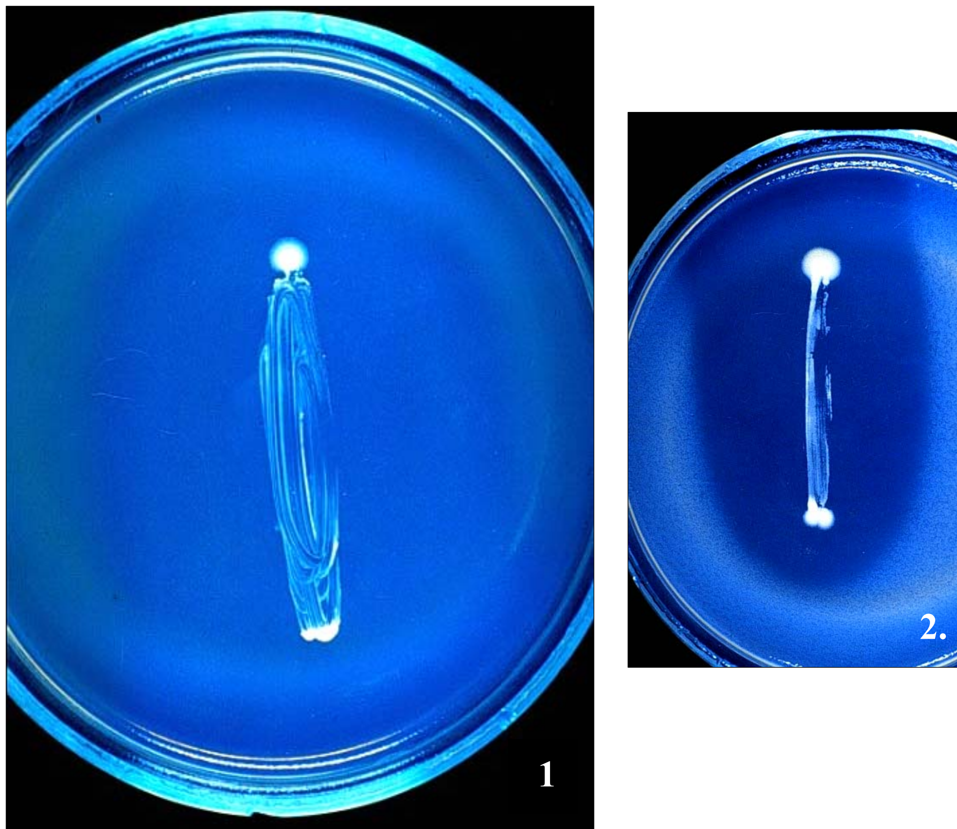


Fig. 15. Antagonistic effect of epiphytic isolate HIP32 **against *Xanthomonas vesicatoria* strain Xv14 (1.) and *Erwinia amylovora* strain Ea1 (2.)**

4.3. Confirmation of metabolite production of epiphytic isolate HIP32

To confirm that the inhibition effect of the antagonistic epiphytic isolate HIP32 on the pathogens was a result of antibacterial metabolite production of the antagonist or it was a result of other factors such as acidity of the medium around the antagonistic isolate. The tolerance of the pathogen as well as the antagonist to acidic media was determined.

4.3.1. Determination of pH tolerance of the pathogen

The tolerance of *Erwinia amylovora (*Ea1, Ea23*)*, and *Xanthomonas vesicatoria* (SO8, Xv14) to different pH values was determined. The pH value of the medium that tolerated by pathogenic strains was found lower about (pH 5). pH value of culture filtrate of the antagonistic

isolate after 7 days growth in buffered medium was also determined as pH 6.5 – 7.2. Otherwise the antagonist had displayed poor growth below pH 6.0, optimal growth was found at pH 6.5 - 7.2 of the media (potato dextrose broth and Ayer's basal media). Results demonstrated the sensitivity of isolate HIP32 to acidity of the media at (lower pH), also growth in buffered medium was optimum and gave indication of metabolite production by its inhibitory action against indicator pathogens in agar diffusion test as below. These results indicted that antagonistic effect of isolate HIP32 is not due to pH changes but to metabolite production in the media.

4.3.2. Effect of culture filtrate of isolate HIP32 by agar diffusion test

Culture filtrate of antagonistic isolate HIP32 was separated by cultivating it for 7days on Ayer's basal medium and/or PDB medium and tested against some plant pathogenic bacterial strains. It was demonstrated that culture filtrate of isolate HIP32 contained antagonistic metabolites effective against the indicator pathogens (Fig. 16.). Ayer's basal medium was found more effective than PDB in stimulating the activity of antagonistic isolate HIP32 and production of metabolites.

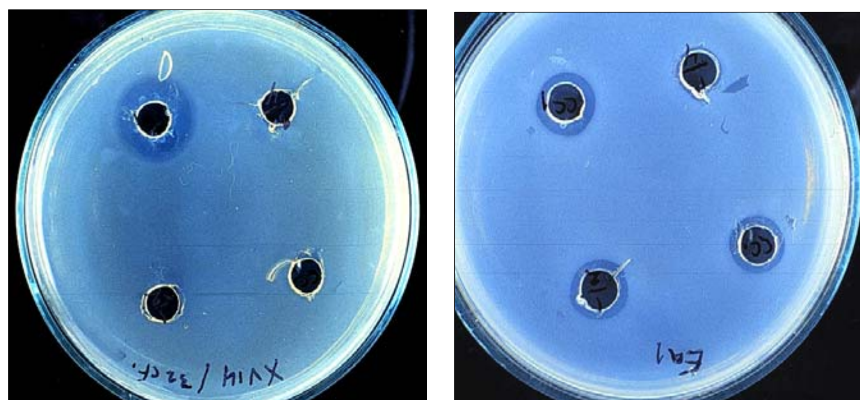


Fig. 16. Reduction of growth of some bacterial pathogens by culture filtrate of isolate HIP32 at different degrees of dilutions. (Left = *Xanthomonas vesicatoria* strain Xv14. Right.= *Erwinia amylovora* strain Ea1).(dilutions in photo: upright; 50%, downright; 25%, down left 12.5%, uplift; control)

4.3.3. Effect of culture filtrate of isolate HIP32 by poisoned agar plate

The effect of the metabolites produced by isolate HIP32 in its culture filtrate was confirmed by determining the minimal inhibitory concentration (MIC) by the inhibitory concentrations culture filtrate (CF) in PDA medium at dilutions of 1:50, 1:25 and 1:12.5 and plated to the indicator bacterial pathogens by double layer agar method. After 7–days of incubation the survived CFU of the indicator strains of *Erwinia amylovora* (Ea1) and (Ea23), also *Xanthomonas vesicatoria* strain (Xv14) and *Clavibacter michiganensis* subsp. *michiganensis* (Cm3) were counted. At 1:50 concentration MIC displayed stronger effect but at 1:25 and 1:12.5 concentrations of CF to the PDA medium MIC was weak or did not work in comparison to control which was consisted of pure cultures of the pathogenic strain only (Fig. 17. and 18.)

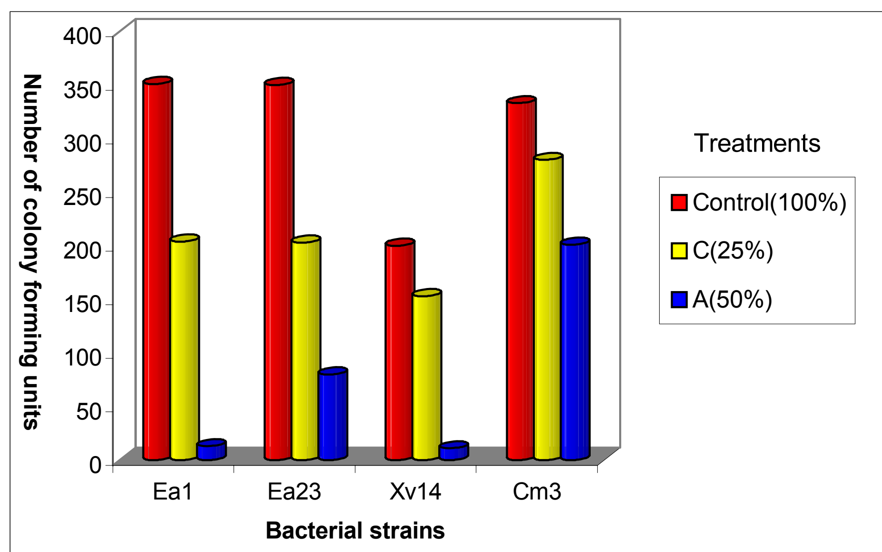


Fig. 17. Reduction of colony forming units of some pathogens* by culture filtrate of isolate HIP32 at different of dilutions**:

Strains ; Ea1 and Ea23 = *Erwinia amylovora*, Xv14 = *Xanthomonas vesicatoria*, Cm3 = *Clavibacter michiganensis* subsp. *michiganensis
****1:50 = 1:2 concentration., 1:25 = 1:4 concentration. of CF to PDA medium**

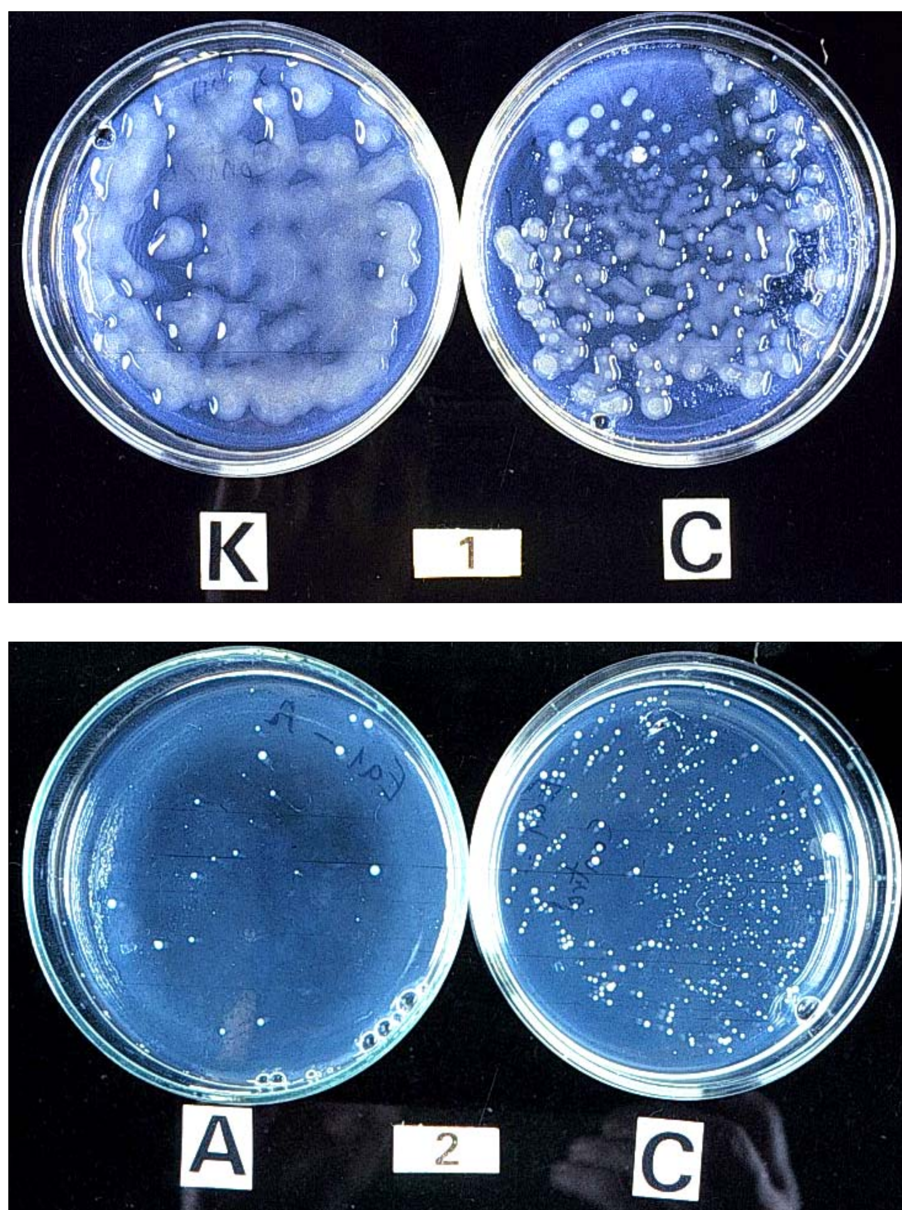


Fig. 18. Reduction of colony forming units of some bacterial pathogens on poisoned agar plate prepared by culture filtrate of isolate HIP32
(Photo 1.) = Colony size of *Xanthomonas vesicatoria* (Xv14) on PDA + CF of epiphytic isolate HIP32 at 25% and PDA medium as control (K) (C),
(Photo 2.) = Reduction of CFU of *Erwinia amylovora* strain (Ea1) on PDA medium +(CF) at 50% (A) and on PDA as control (C)

4.4. Time dependence of quantity of metabolite production of isolate HIP32

Double layer agar and streaking methods were used for some strains of plant pathogenic bacteria against isolate HIP32 to determine time dependency of the antagonistic effect. Results demonstrated that the metabolite activity of the epiphytic isolate HIP32 as determined by inhibition zones is influenced by the time of cultivation. Inhibition zones (mm) demonstrated that antagonistic activity of isolate HIP32 was stronger on the 6th and 8th days in all treatments. It was not determined whether at longer intervals the antagonist produced more or other metabolites as quantity or quality changes (Fig. 19. and 20.).

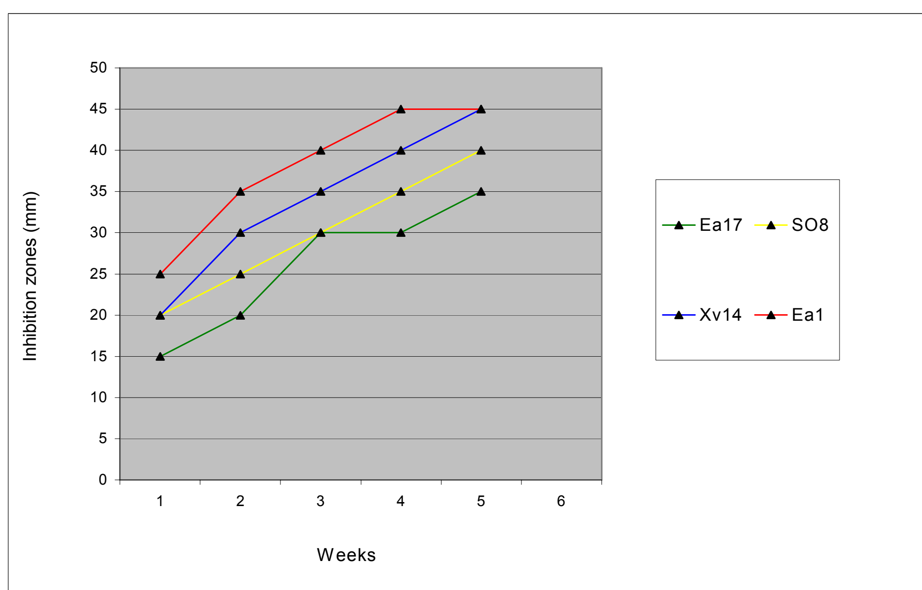


Fig. 19. Time dependence activity of antagonistic isolate HIP32 towards pathogenic strains of *Erwinia amylovora* (Ea1 and Ea23) and *Xanthomonas vesicatoria* (Xv14 and SO8)

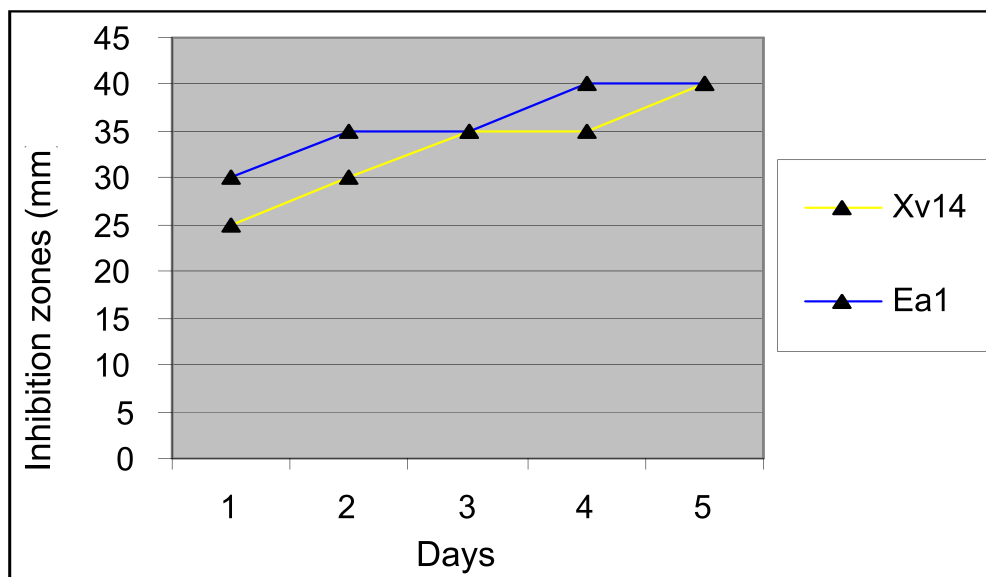


Fig. 20. Time dependence activity of isolate HIP32 towards *Erwinia amylovora* strain Ea1 and *Xanthomonas vesicatoria* strain Xv14

4.5. Persistence of epiphytic isolate HIP32 in apple trees

It was demonstrated that isolate HIP32 has been successfully persisted and established from May to August in it's original host, apple cultivar 'Starking' after it was sprayed on leaves using suspension (at 10^7 cfu/ml^{-1}) in three independent experiments (Table 8. and Fig. 21.). During 4-weeks of samplings summer was more optimal for persistence than winter and spring time. Not only persistence but also multiplication of epiphytic isolate HIP32 was found to be abundant during the temperate period in May and June (temperature above 30°C). Growth was abundant with a larger colony size and yellow color when reisolation of HIP32 was done on selective medium.

Table 8. Persistence of epiphytic isolate HIP32 in apple trees

Week	Number of colony forming units /cm^2 leaf area		
	May	June	July-August
0	4.33	4.79	5.54
1	5.27	5.81	6.04
2	5.66	6.08	7.18
3	5.75	6.29	7.34
4	6.34	6.68	7.61

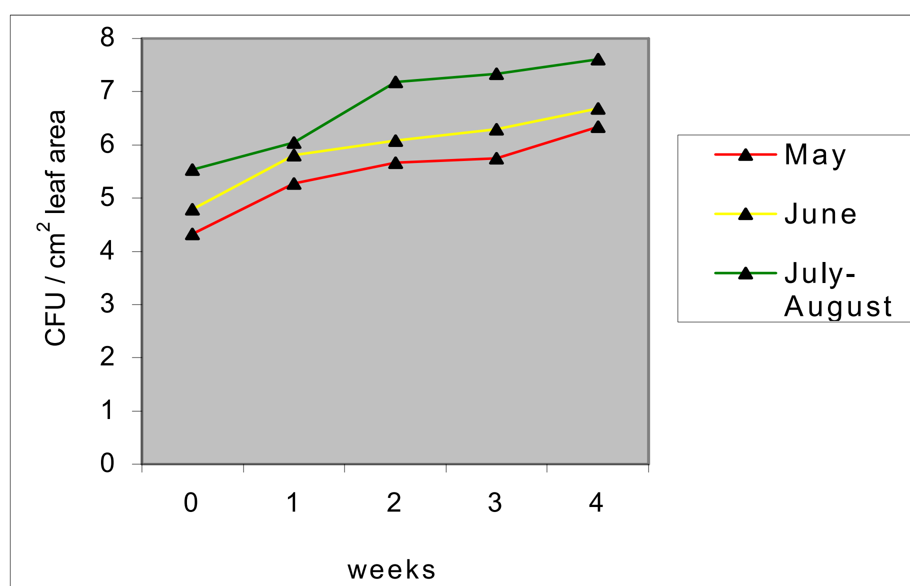


Fig. 21. Seasonal establishment of epiphytic isolate HIP32

4.6. Isolation of antagonistic bacteria from rhizosphere

The purpose of this work was to isolate soil antagonists to soil-borne plant pathogenic bacteria especially to *Clavibacter michiganensis* subsp. *michiganensis* and *Agrobacterium vitis*. Screening for antagonists from soil samples collected from different places in Hungary and Libya has been carried out during the course of this study. Results demonstrated that among the rhizosphere microflora there are numerous bacterial species antagonistic to phytopathogenic bacteria. From a large numbers of isolates in 25 total soil samples there were about 850 isolates including many isolates that had wide spectrum effects and display clear inhibition zones in most treatments done by the double agar layer or streaking methods. Besides *Clavibacter*

michiganensis subsp. *michiganensis* and *Agrobacterium vitis* the effectivity of antagonists was also confirmed for other plant pathogens (Table 9. and Fig. 22.).

Table 9. **Bacterial antagonists isolated from the rhizosphere towards some pathogenic bacterial species**

bacterial species	Sum of No. of antagonistic isolates	Percentage of antagonists
Agrobacterium vitis	130	15.3
Clavibacter michiganensis subsp. *michiganensis*	145	17.1
Pseudomonas syringae pv. *syringae*	128	15.0
Erwinia carotovora subsp. *carotovora*	153	18.0
Xanthomonas vesicatoria	123	14.5
Erwinia amylovora	104	12.2

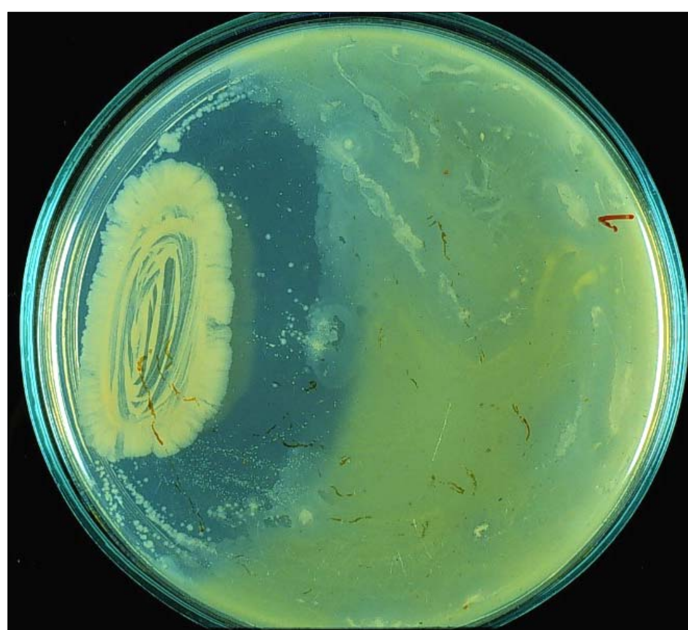


Fig. 22. Effect of soil isolate HIR225 towards *Xanthomonas vesicatoria* strain SO8 by double layer method

4.6.1. Preliminary selection of soil antagonistic bacterial isolates

Several antagonists were found to be effective against most plant pathogenic bacterial species tested as described above. The most effective isolates against *Erwinia amylovora, Erwinia carotovora* subsp. *carotovora, Xanthomonas vesicatoria, Pseudomonas. syringae* pv. *syringae, Clavibacter michiganensis* subsp. *michiganensis* and *Agrobacterium vitis* were selected depending on their effect assayed by the double agar layer method. According to the spectra of effectivity of the antagonists on the tested plant pathogenic species it was possible to divide these antagonists into different categories; A, B, C and D (Table 10.).

Table 10. **Effects of selected antagonistic isolates from soil samples against some plant pathogenic bacteria as assayed by the double layer agar method**

Selected antagonistic Isolates*	*Effect on pathogenic bacteria***						Categories***
224, **225,** 309, 424,427, 704,712, 916, 1013, 1108, 1113, 1301,1311,1314,1318, 1417, 1813, 1901,1907,	Blue	Red	Green	Brown	Yellow	Violet	**A**
124, 804,811,814, , 1703, , 2308, 308,	Blue	Red	Green	Brown	Yellow		**B**
108, 113, 121, 122, 515, 528, 610, 1510, 1601, 2014, 2124, 2401,2408	Blue	Red	Green			Violet	**C**
2207, 2501		Red	Green		Yellow		**D**

, * = Codes given to soil sample number + isolate number
** Blue = *Erwinia carotovora* subsp. *carotovora* (10), Red = *Agrobacterium vitis*, Green = *Clavibacter michiganensis* subsp. *michiganensis*, Brown = *Pseudomonas syringae* pv. *syringae* , Yellow = *Xanthomonas vesicatoria*, violet =*Erwinia amylovora*, *** = A, B, C, D, are categories of effectivity of the antagonistic isolates from higher(A) to lower effect (D).

4.6.2. Effectivity of selected soil antagonists against some plant pathogens

Results of the above mentioned tests showed that a group of antagonistic isolates was recognized from the collection of isolates effective against most plant pathogenic species tested in this study, especially *Agrobacterium vitis* and *Clavibacter michiganensis* subsp. *michiganensi*s using inhibition zone measurements (Table 11. and 13.).

Many antagonistic isolates demonstrated strong and clear inhibition zones, others showed wide spectrum effect to most pathogenic strains tested. From these isolates that displayed different antagonistic effects on the basis of *in vitro* antagonistic tests isolate 225 was selected for its strong inhibitory and wide spectrum effects to continue further experimental work and coded as Hungarian rhizosphere isolate HIR225.

Soil isolate HIR225 was also tested against some formae speciales of *Fusarium oxysporum* and *Geotrichum candidum* after 2-3 days incubation of the soil isolate. Evaluated the effectivity by measuring the distance between the antagonist and the growth of the fungus (Table 12.).

From numerous isolates that were antagonistic against most strains of bacterial pathogens also their spectra according to inhibition zones isolate HIR225 was selected for further studies and identified because it was the best in its effects.

Table 11. Antagonistic effects of some soil antagonistic isolates against *Clavibacter michiganensis* subsp. *michiganensis* strain Cm3

Code of soil antagonists	Inhibition zone (mm)	Code of soil antagonists	Inhibition zone (mm)
310	10	**113**	25
321	10	**2014**	25
326	10	**108**	35
121	15	**122**	30
224	15	**528**	35
311	15	**610**	35
427	15	**704**	35
1108	15	**811**	35
1204	15	**814**	35
1314	15	**916**	35
1404	15	**1013**	35
1415	15	**1113**	35
1715	15	**1318**	35
1807	15	**1417**	35
1901	15	**1703**	35
1907	15	**1710**	35
2003	15	**1813**	35
2110	15	**2308**	35
2207	15	**2401**	35
306	18	**2408**	35
322	18	**2501**	35
309	18	**308**	40
115	20	**225**	**45**
515	25	**2124**	45

Table 12. Spectra of antagonistic effects of soil isolate HIR225 towards some fungal species

Pathogenic fungi	**Inhibition zones (mm)**
Fusarium oxysporum f. sp. *cyclaminis*	35
Fusarium oxysporum f. sp. *dianthi*	30
Fusarium oxysporum (-)	30
Geotrichum candidum	35

Table 13. Antagonistic effects of some soil isolates against *Agrobacterium vitis* (Av66)

Code of soil antagonists	**Inhibition zone (mm)**	**Code of soil antagonists**	**Inhibition zone (mm)**
310	10	**515**	25
321	10	**2014**	25
326	10	**122**	30
224	15	**108**	35
121	15	**528**	35
427	15	**610**	35
311	15	**704**	35
1108	15	**811**	35
1204	15	**814**	35
1314	15	**916**	35
1404	15	**1013**	35
1415	15	**1113**	35
1715	15	**1318**	35
1807	15	**1417**	35
1901	15	**1703**	35
1907	15	**1710**	35
2003	15	**1813**	35
2110	15	**2308**	35
2207	15	**2401**	35
306	18	**2408**	35
309	18	**2501**	35
322	18	**308**	40
115	20	**225**	**40**
113	25	**2124**	40

4.7. Sensitivity of the tested pathogens and antagonists to different antibiotics

Knowledge of sensitivity to antibiotics of the epiphytic (HIP32) and soil (HIR225) isolates and the plant pathogenic bacterial strains of *Clavibacter michiganensis* subsp. *michiganensis* (Cm3) and *Xanthomonas vesicatoria* (SO8, Xv14) for preparation of selective media was necessary. Results gave a comparison of sensitivity to antibiotics, which indicated that *Xanthomonas* strains Xv14, SO8 were resistant to **Nitrofurantoin** at (100ppm) in comparison to antagonistic strains that were inhibited by these antibiotics. Also *Clavibacter michiganensis*

subsp. *michiganensis* was resistant to **Lincomycin** (at 50 and 100ppm) in comparing to antagonistic strains. (HIP32 and HIR225). These two antibiotics were chosen to be incorporate in selective media for reisolation of the pathogenic strains in order to evaluate the effectivity of antagonists in soil experimental studies (Table 14.).

Table 14. Antibiotic sensitivity of different plant pathogenic bacterial species and epiphytic antagonistic isolate (HIP32) and soil isolate (HIR225)

Antibiotic / active substance	**HIP32***	**SO8**	**Xv14**	**Cm3**	HIR225
Penicillin / 3 I.U.	R **	MS	R	R	S
Methicillin / 20µg	**R**	R	R	R	S
Ampicillin / 20µg	R	R	R	R	S
Streptomycin / 30µg	S	S	S	S	S
Neomycin / 100µg	S	MS	S	S	S
Kanamycin / 30µg	S	S	S	S	S
Chloramphenicol / 30µg	S	S	S	S	S
Tetracycline / 30µg	S	S	S	S	S
Oxytetracycline / 30µg	MS	S	S	S	MS
Oleandomycin / 30µg	R	MS	MS	S	MS
Erythromycin / 10µg	R	MS	R	S	S
Polymyxin B / 15µg	S	S	S	R	S
Cephalothin / 10µg	R	R	R	R	**MS**
Nystatin / 100 I. U.	R	R	MS	R	MS
Nitrofurantoin/ 100ppm	**S**	R	R	**MS**	S
Trimethoprim / 100ppm	R	R	R	S	S
Lincomycin / 50, 100ppm	S	MS	N t.	R	S

*(32) = epiphytic isolate, Cm3= *Clavibacter michiganensis* subsp. *michiganensis.*
Xv14 = *Xanthomonas vesicatoria* from pepper, SO8 = *Xanthomonas vesicatoria* from tomato, 225 =soil isolate
** Inhibition effect: Sensitive (S > 20mm), Moderately sensitive (MS 11-20mm), Resistant (R < 11), . N t. = not tested

4.8. Description of the antagonistic isolates

For selection of biological control agents it is important to verify the non-pathogenic characters of the tested isolates in plants and it is especially essential to analyze the saprophytic characters of antagonistic isolates.

4.8.1. Confirmations of saprophytic characters of selected isolates

Different methods were applied on different plant species in order to confirm the saprophytic characters of the selected antagonistic isolates

Plant inoculation of epiphytic and soil isolates HIP32 and HIR225 were inoculated to tomato, pepper, apple, and tobacco plants but caused no symptoms or damages so they did not show any pathogenic ability in these plants during 3-7 days incubation.

Hypersensitive reaction of tobacco plants were inoculated with antagonistic isolates HIP32 and HIR225 at different concentrations, plants were evaluated for symptoms at 24, 48 and 72 hrs after inoculations. No hypersensitive reaction was recorded in tobacco leaves.

Soft rot test inoculation sites of potato slides were not rotted and pectolytic activity was not recorded for antagonistic isolates, HIP32 and HIR225 after 7 days.

4.8.2. Cultural and morphological characteristics of isolate 32

Cultural characterization of isolate 32 showed that it is Gram-negative by the KOH test. Colony type differed according to medium type and age of the isolate about (1 week or more), colonies had no fluorescence pigments, could grow well on Nutrient, PDA, King B and YDC media at pH 6.5 – 7.0.

Characteristics of colonies grown on PDA, King B and NA media for 1-week was observed by stereo microscope (10- and 20X magnification), for isolate HIP32 (Table 15.). After heating at 80˚C for different periods of time, no spore forming and no growth was recorded from the killed vegetative cell suspension onto nutrient or PDA media.

Flagellation was demonstrated by scanning electron microscope cells of isolate HIP32 were short rods (0.56 and 0.72 μm), peritrichously flagellated, and single or in chains (Fig. 23. and 24.)

Table 15. Colony types of antagonistic isolate HIP32 on different media

Characteristics of the colonies (7 days incubation)

<table>
<tr><th>Medium</th><th>Shape</th><th>Color</th><th>Size(mm)</th><th>Form</th></tr>
<tr><td>Nutrient</td><td>Rigid-circular</td><td rowspan="2">Pale white-yellow</td><td>Small
1-2.5</td><td>Domed-entire</td></tr>
<tr><td>PDA</td><td rowspan="2">Irregular wavy</td><td rowspan="2">Medium
2.5-3.5</td><td>Abundantly Mucoid, domed</td></tr>
<tr><td>King B</td><td>Creamy-yellow</td><td>Non-mucoid raised</td></tr>
<tr><td>YDC</td><td>Irregular-restricted</td><td>waxy white</td><td>Large
>4</td><td>Curly-flat</td></tr>
</table>

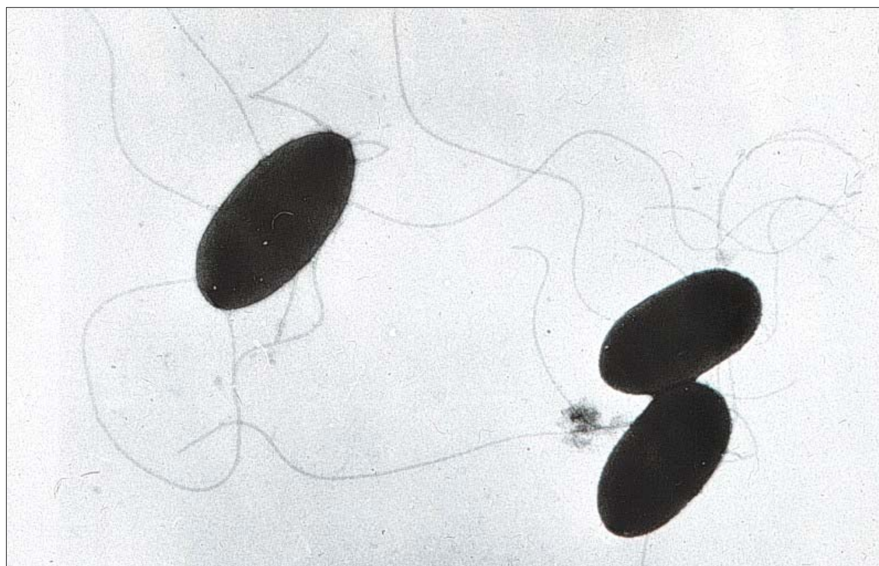


Fig. 23. Cell morphology of *Pantoea agglomerans* strain HIP32 under electron microscope (10000 nm) (cells are short rods, 0.56 and 0.72 µm, single or in chain and peritrichously flagellated)

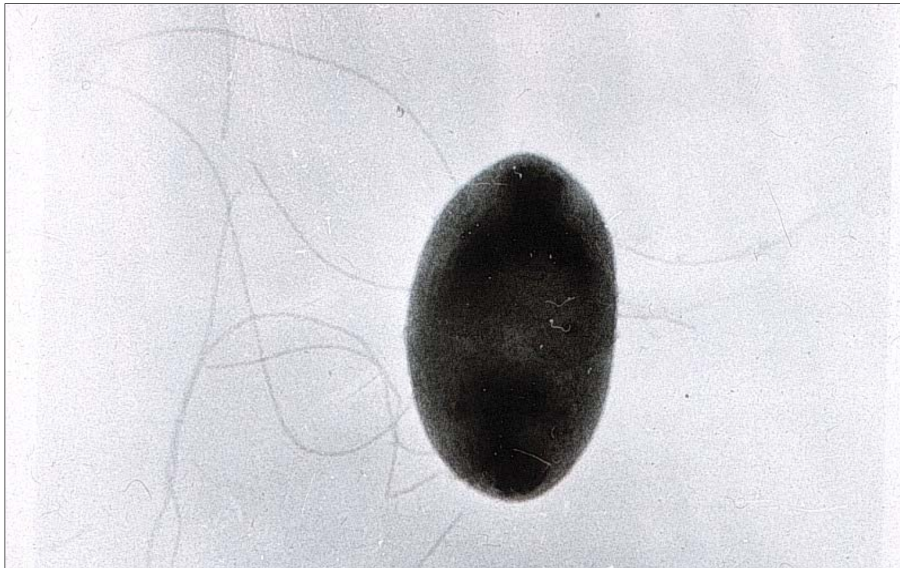


Fig. 24. Cell morphology of *Pantoea agglomerans* strain HIP32 was studied under electron microscope (21560 nm) (cells are short rods, 0.56 and 0.72 µm, single or in chain and peritrichously flagellated).

Photo: **Susanne Richter from Federal Office and Research Center for Agriculture, Wien, Aust.**

4.8.3. Biochemical characteristics of isolate HIP32

As indicated in (Table 23) oxidase reaction was negative, glucose utilization by fermentation was positive, motile, and facultative anaerobic tests suggested that isolate HIP32 belongs to the family *Enterobacteriaceae*. Also *Enterobacter* species are negative for H_2S, indole, phenylalanine and β-Galactosidase tests, reduce nitrates which suggesting that isolate HIP32 belongs to the family *Enterobacteriaceae* as *Enterobacter* spp.

For determination of the species level, API 20 E tests were chosen and results evaluated by the identification software API PLUS. The results in (Table 16.) indicated that isolate HIP32 is *Enterobacter agglomerans* the synonym of *Erwinia herbicola*. Other biochemical characteristics of isolate HIP32 such as gas formation from glucose that was positive indicating that this isolate belongs to the aerogenic biogroup. The main characteristics differentiating within this biogroup such as the negative indole test and the positive Voges-Proskauer tests were achieved. Therefore isolate HIP32 (*Enterobacter agglomerans)* was assigned to the aerogenic biogroup G1. Recently the binomial *Pantoea agglomerans* comb. Nov. is used for this bacterium (Ewing and Fife, 1972, Gavini et al., 1989).

Table 16. Biochemical characteristics of isolate HIP32 as assayed on different substrates by API 20 E tests

Code of test	Substrates	Results
ONPG	Ortho-nitro-phenyl-galactoside	+
ADH	Arginine	-
LDC	Lysine	-
ODC	Ornithine	_
CIT	Sodium citrate	+
H_2S	Sodium thiosulphate	_
URE	Urea	_
TDA	Tryptophane	_
IND	Tryptophane	_
VP	Sodium pyruvate	+
GEL	Kohn's gelatin	_
GLU	Glucose	+
MAN	Mannitol	+
INO	Inositol	+
SOR	Sorbitol	+
RHA	Rhamnose	+
SAC	Sucrose	+
MEL	Melibiose	+
AMY	Amygdalin	+
ARA	Arabinose	+
OX	Tetramethyl-p-phenylene-diamine dihydrochloride	_
NO_3	NO_2	+
Motility	by electron microscope	motile
MAC	Growth on Mac Conkey medium	+
Gas from glucose	Glucose	+

4.8.4. Cultural and morphological characteristics of soil isolate

Characteristics of soil isolate HIR225 were studied and results demonstrated that it has saprophytic characters and its colonies are non-fluorescent, non-pigmented. As observed under electron microscope, cells are short rods (0.7 – 0.8 by 2.0 – 3.0μ), peritrichously flagellated, and cells are in chains (Figs. 25. and Fig. 26.). Also, it was confirmed that the bacterium is sporeforming and it could recover following heating periods of 80 ^{o}C and 110°C for more than half an hour.

Antagonistic soil isolate HIR225 was tested on different media. Growth was optimal and faster on PDA medium than on Nutrient, King B and Yeast dextrose chalk agar following growth for 1–3 days, colonies on PDA were white, irregular, large, mucoid abundantly, wavy, and flat while they developed slowly on other media. Growth in Nutrient broth could tolerate the optimal between 30 – 40°C while the maximum tolerated temperature for this strain was 50°C to 55°C and pH. 6.0, 6.5, 7.0. (Table 17.).

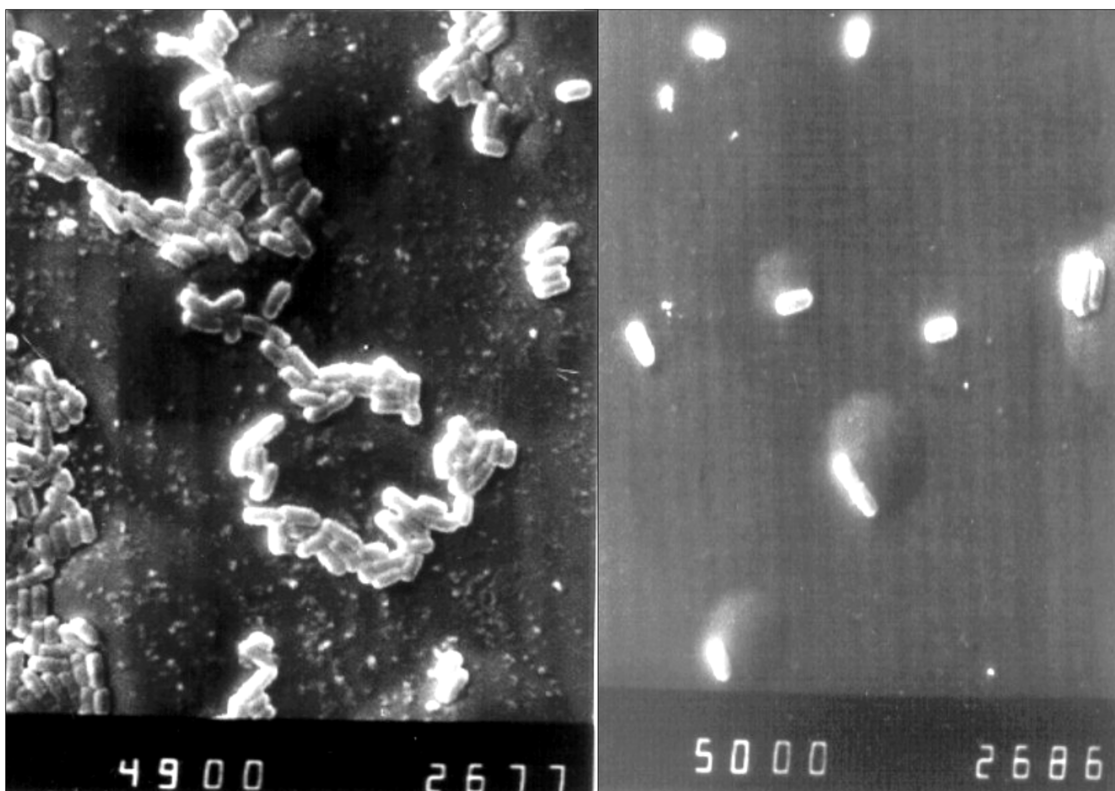


Fig. 25. Cell morphology of soil isolate HIR225 of *Bacillus subtilis* under electron microscope at left: (2450X), right: (2500X)

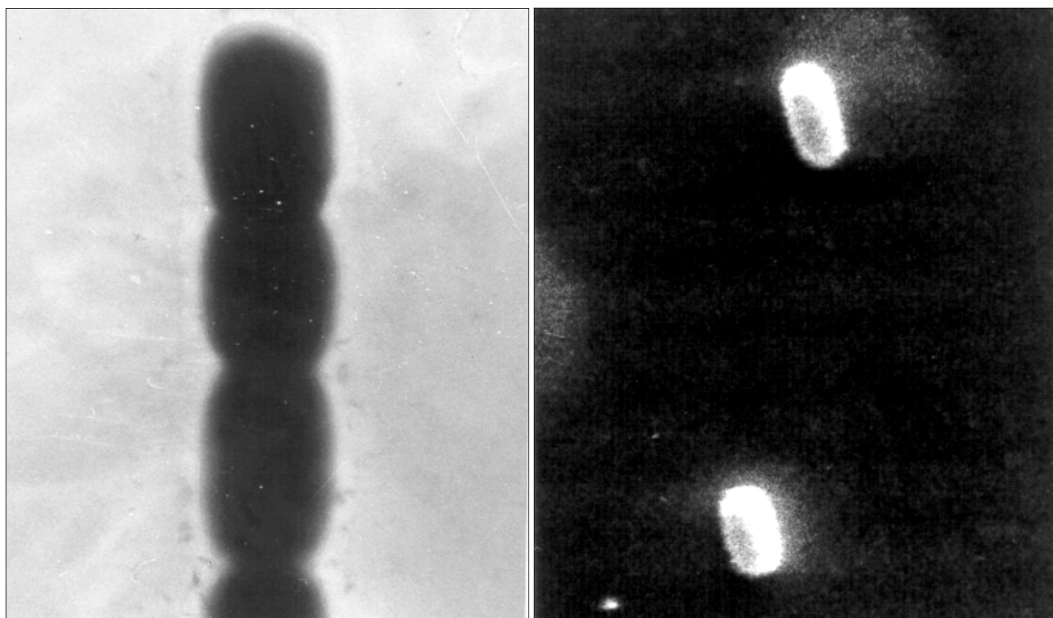


Fig. 26. Cell morphology of *Bacillus subtilis* under electron microscope left: (21600X) and right: (5600X)

Photo: Nagy Barbara, Central Lab. of E.M. in SZIE University, Budapest-Hungary

<u>Table 17.</u> Characters of colony type of antagonistic soil isolate HIR225 on different media

Characteristics of colonies (7 days incubation)				
Media	**Shape**	**Color**	**Size/mm**	**Form**
PDA			**Large >4**	**Mucoid, Wavy-flat**

<table>
<tr><td>King B</td><td rowspan="3">Irregular</td><td>White</td><td rowspan="3">Medium
2 – 3.5</td><td>Non-mucoid-
Wavy-flat</td></tr>
<tr><td>YDC</td><td rowspan="2">Waxy white</td><td rowspan="2">Curly-flat</td></tr>
<tr><td>Nutrient</td></tr>
</table>

4.8.5. Biochemical characterization

Results of biochemical tests were summerized in table 18**:**

Table 18. Biochemical characteristics of soil isolate HIR225

Tests	Results
Sporeforming	+
Gram reaction	+
Cytochrome oxidase,	+
Catalase	++
Nitrate reduction	+
Starch hydrolysis	+
Gelatin hydrolysis	+
Aesculin hydrolysis	++
Indole production	+
H_2S Production on TSI medium	-
Sodium citrate utilization	+
Casein hydrolysis	+
Voges Proskauer	+
Urease production	-
Deamination of phenylalanine	-
Malonate utilization	-
Arginine dihydrolase	-
glucose /fermentation/oxidation	weak
Gas from glucose	-
PH	5.8 - 6.8
Growth at temperature	30 - 50, 55°C

According to results of the above mentioned tests and the biochemical tests in (Table18) the soil isolate HIR225 investigated was found to belong to the family *Bacilliaceae* and, recognized as *Bacillus subtilis.*

The antibiotic effect on the soil microflora was studied and compared with the effect of antibiotics on plant pathogenic bacterial species. Data were essential for following the cell numbers of the pathogens on selective media. Results indicated that there were clear effects of antibiotics on reduction of growth of soil microflora (Table 19.). According to the sensitivity tests, among the antibiotics Nitrofurantoin was selected for reisolation of *Xanthomonas vesicatoria* strain SO8 and Lincomycin was selected for reisolation of *Clavibacter michiganensis* subsp. *michiganensis* strain Cm3.

Table 19. Sensitivity of soil type Őrbottyán used for soil treatments to different antibiotics

Antibiotics	Effect on soil sample
Nystatin	S*
Methicillin	S
Ampicillin	S
Nitrofurantoin	S
Trimethoprim	S
Lincomycin	S

*S = Sensitive to antibiotics

Further studies on soil strain HIR225 (*Bacillus subtilis)* have been initiated by conducting soil inoculations to investigate the antagonistic characters *in vivo.*

4.9. Quantitative analysis of survival of pathogens in the soil under the influence of the antagonists

The biocontrol effects of *Pantoea agglomerans* and *Bacillus subtilis* were evaluated against some plant pathogenic species using a soil sample from Őrbottyán, Hungary. Numbers of colony forming units (CFU) of pathogens in the presence of antagonists in the soil were followed in separated experiments by monitoring the persistence of indicator pathogens for several weeks. The sterile fallow sandy type soil was inoculated with a suspension (15ml water capacity + 15ml ”$2x10^8$cfu/ml^{-1}”) of *Xanthomonas vesicatoria* (SO8) and *Clavibacter michiganensis* subsp.

michiganensis (Cm3) and antagonistic strains *Pantoea agglomerans* or *Bacillus subtilis* at the same time. Soil water content (soil capacity) was adujsted when neccesary to (30ml/100g^{-1}soil).

Sampling was started at 0-time and continued until the 4th week. The numbers of colony forming units of the pathogens were counted weekly. Results demonstrated that antagonistic

strains *Pantoea agglomerans* (HIP32) and *Bacillus subtilis* (HIR225) were effectively reduce growth of *Xanthomonas vesicatoria* strain SO8, which was recovered and reisolated from treated soil (Fig. 27.). In another independent experiment it was clarified that these antagonistic strains are moderately effective against *Clavibacter michiganensis* subsp. *michiganensis,* which was also recovered and reisolated from treated soils (Fig. 28.). The antagonistic effect was more pronounced after 3 weeks samplings.

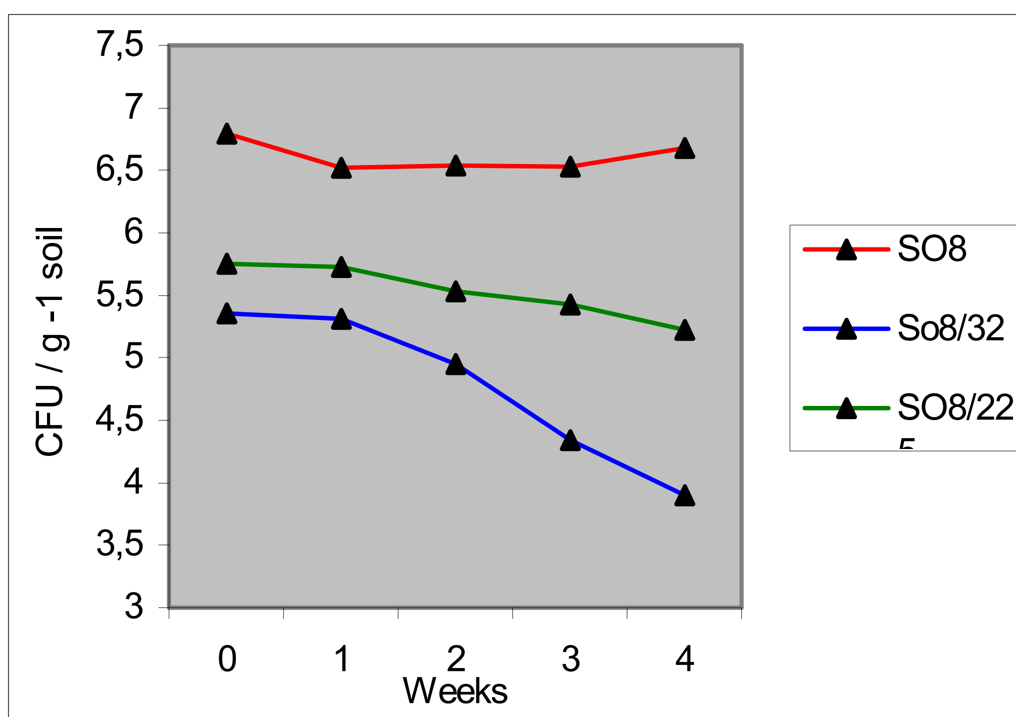


Fig. 27. Recovery of *Xanthomonas vesicatoria* strain SO8 from soil treated with *Bacillus subtilis* (HIR225) and *Pantoea agglomerans* (HIP32)

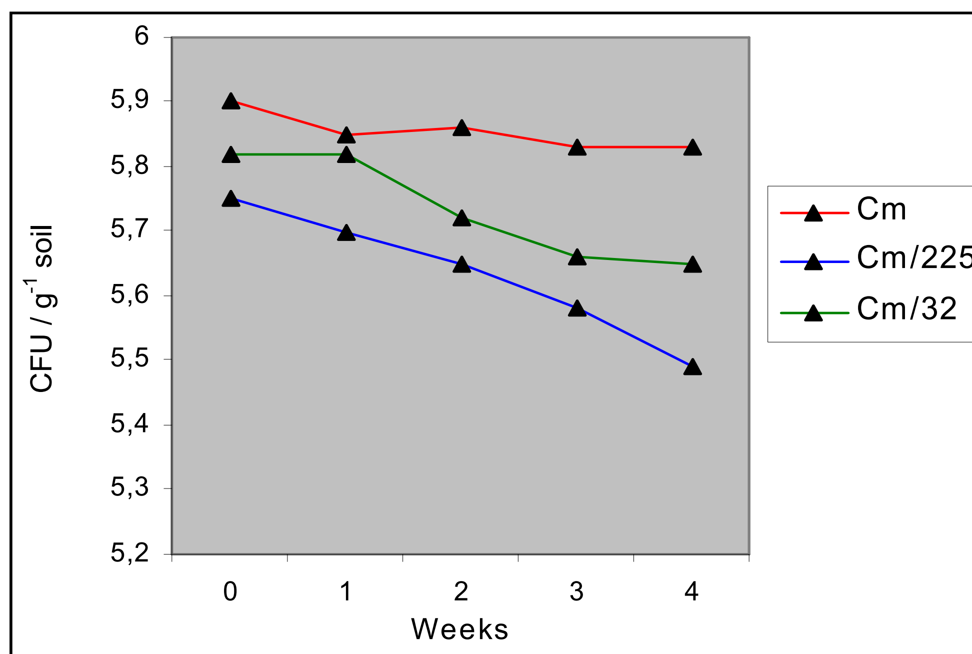


Fig. 28. Recovery of *Clavibacter michiganensis* subsp. *michiganensis* strain Cm3 from soil treated with *Bacillus subtilis* (HIR225) and *Pantoea agglomerans* (HIP32)

4.10. Antagonistic effects of *Pantoea agglomerans*

Utilization of *Pantoea agglomerans* as biological control agent against fire blight disease of pomaceous plants caused by *Erwinia amylovora* and bacterial spot disease of tomato and pepper caused by *Xanthomonas vesicatoria* was demonstrated. The utility of this antagonistic strain was detected in different experiments using different plant organs such as: leaves, fruits, flowers and intact plant seedlings.

4.10.1. Fire blight disease reduction in apple leaf discs treated with *Pantoea agglomerans*

For estimation of capability of *Pantoea agglomerans* to reduce fire blight disease severity in leaf-discs of different *Malus domestica* cultivars. Symptoms were judged according to leaf spot numbers and area and gradual browning of the leaf tissues using a 5-degree scale. Pre-

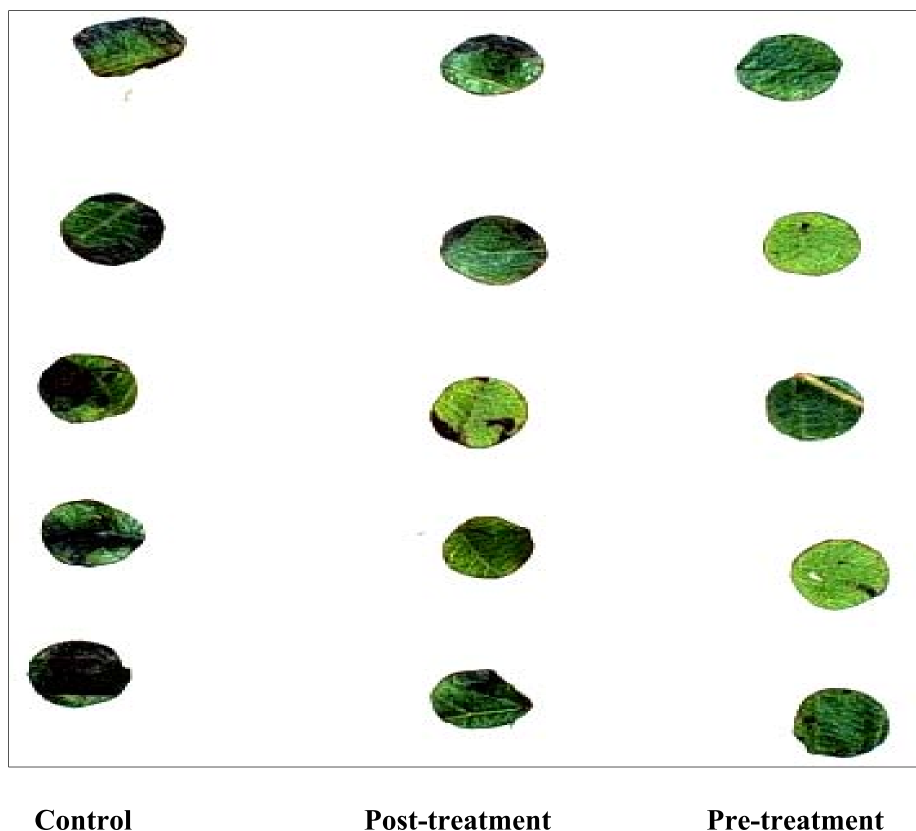
treatment (applying *Pantoea agglomerans* 24hr before pathogen inoculation) was generally more effective than post-treatment, where the antagonist was inoculated 24hr after the pathogen, although post-treatment was effective in case of some cultivars (Table 20. and Fig. 29.).

Table 28. Effect of *Pantoea agglomerans* on reduction of fire blight disease of different apple cultivars by leaf discs method

<table>
<tr><th rowspan="2">Cultivars</th><th colspan="4">Percentage of disease reduction %</th></tr>
<tr><th>Pre-treatment</th><th>Effectivity</th><th>Post-treatment</th><th>Effectivity</th></tr>
<tr><td>Spartan*</td><td rowspan="4">85-98</td><td rowspan="4">H</td><td rowspan="4">34-51</td><td rowspan="4">M</td></tr>
<tr><td>Prima</td></tr>
<tr><td>Liberty</td></tr>
<tr><td>Starking</td></tr>
<tr><td>Mutsu (9M)</td><td rowspan="2">78-96</td><td rowspan="2">H</td><td>34-49</td><td>L**</td></tr>
<tr><td>Idared (M4)</td><td>0</td><td>NE</td></tr>
<tr><td>Granny Smith</td><td>66-76</td><td>H</td><td>34-51</td><td>M</td></tr>
<tr><td>Idared (M9)</td><td rowspan="3">50-54</td><td rowspan="3">M</td><td rowspan="3">0</td><td rowspan="4">NE</td></tr>
<tr><td>Freedom (MM106)</td></tr>
<tr><td>Csányi</td></tr>
<tr><td>Control</td><td>0</td><td>NE</td><td>0</td></tr>
</table>

*** catagories are significantly different at 5%**

****L = Low effectivity (10 - 49%), M= Moderately effective (50 - 64%), H= Highly effective (65 - 100 %), NE= No effect of antagonist or errror.**



Control **Post-treatment** **Pre-treatment**

Fig. 29. Fire blight disease reduction on apple leaf discs pre-treated and post-treated with strain HIP32 of *Pantoea agglomerans* comparing to control

4.10.2 Reduction of fire blight disease severity by *Pantoea agglomerans* in leaves of some pomaceous plant species

The above mentioned experiment of leaf discs was confirmed by inoculation of entire leaves of *Malus domestica*, *Pyrus communis*, *Cydonia oblonga* and *Cotoneaster sp*. by applying strain HIP32 of *Pantoea agglomerans* 24 hr before and after inoculation with different *Erwinia amylovora* strains: Ea1, Ea17, Ea23, and Ea29. In these experiments we have demonstrated the

possible effectivity of *Pantoea agglomerans* in other hosts of *Erwinia amylovora* while apple leaves served as a control.

Results demonstrated the effectivity of *Pantoea agglomerans* in these plant leaves inoculated with *Erwinia amylovora.* The means of disease reduction in leaves with the antagonist applied before inoculation with the pathogen (pre-treatment) was effective while the protective effect of post-treatment was not significant.

Results demonstrated beyond that the disease reduction in cotoneaster and quince was more effective than in apple and pear (Table 21. and Fig. 30., 31., 32., 33. and 34.).

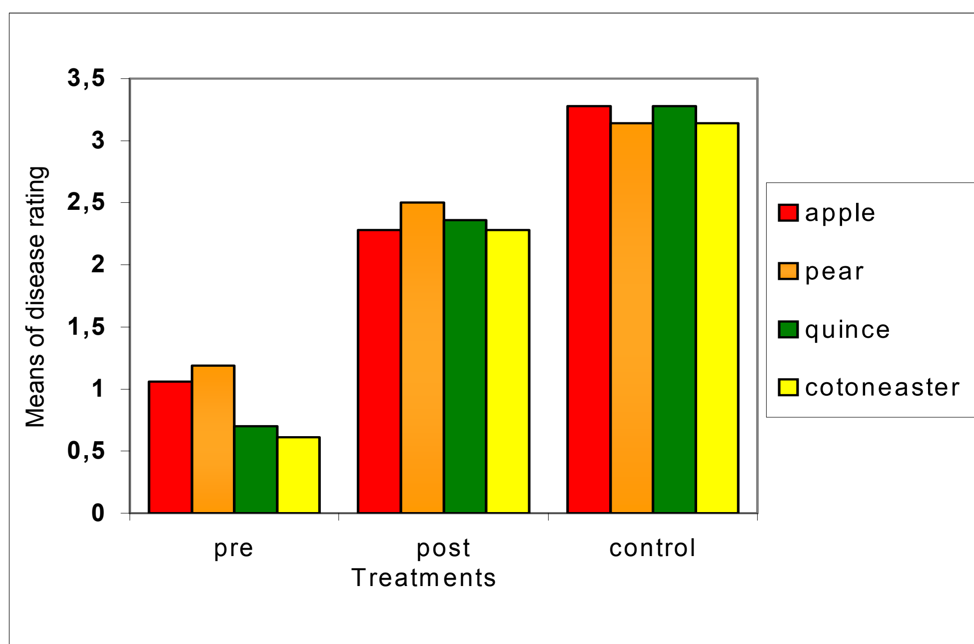


Fig. 30. Means of fire blight disease rating in apple, pear, cotoneaster and quince leaves treated with *Pantoea agglomerans* strain HIP32 before and after inoculation with *Erwinia amylovora* strains

<u>Table 21.</u> Reduction of fire blight disease in apple, pear, cotoneaster, and quince leaves treated by *Pantoea agglomerans* before and after the inoculation with *Erwinia amylovora*

Treatments		apple	pear	cotoneaster	Quince
Pre-treatment					
Replicates	**1**	1.17	0.83	0.50	0.67
	2	0.83	1.67	0.50	0.67
	3	0.33	1.33	0.50	0.83
	4	1.17	1.33	1.17	0.50
	5	1.83	1.00	0.33	0.50
	6	1.00	1.00	0.67	1.00
Mean of disease rating		**1.06**	**1.19**	**0.61**	**0.70**
	Post-treatment				
Replicates	**1**	3.50	2.50	1.83	2.17
	2	3.50	2.00	1.83	1.83
	3	2.67	3.17	2.17	2.50
	4	3.33	1.83	2.50	1.67
	5	3.33	2.50	2.67	2.50
	6	3.33	3.00	2.67	3.50
Mean of disease rating		**2.28**	**2.50**	**2.28**	**2.36**
Control					
Replicates	**1**	3.50	3.00	2.67	3.17
	2	3.50	3.67	3.00	2.67
	3	2.67	3.50	3.00	3.17
	4	3.33	3.33	3.67	3.50
	5	3.33	2.50	3.83	3.50
	6	3.33	2.83	2.67	3.67
Mean of disease rating		**3.28**	**3.14**	**3.14**	**3.28**

Pre-treatment **Post-treatment** **Control**

Fig. 31. Comparison of pre-treatment and post-treatment by strain HIP32 of *Pantoea agglomerans* on reduction of fire blight disease on apple leaves to control treated with *Erwinia amylovora* strain Ea1.

Pre-treatment Post-treatment

Control

Fig. 32. Comparison of pre-treatment and post-treatment by *Pantoea agglomerans* on reduction of fire blight disease on cotoneaster leaves to control inoculated with *Erwinia amylovora* strain Ea29

Pre-treatment Post-treatment Control

Fig. 33. Comparison of pre-treatment and post-treatment by *Pantoea agglomerans* on reduction of fire blight disease on quince leaves to control inoculated with *Erwinia amylovora* strain Ea17

Pre-treatment Post-treatment Control

Fig. 34. Comparison of pre-treatment and post-treatment by strain HIP32 of *Pantoea agglomerans* on reduction of fire blight disease on pear leaves to control inoculated with *Erwinia amylovora* strain Ea23

4.10.3. Reduction of fire blight disease by *Pantoea agglomerans* in fruits and flowers of some pomaceous crops

Garden fruits of *Malus domestica*, *Pyrus communis* and *Cydonia oblonga* were treated with antagonistic strain HIP32 of *Pantoea agglomerans* 24hr before (pre-treatment) and after (post-treatment) inoculation with *Erwinia amylovora* strains *(*Ea1, Ea17, and Ea23) *of* pomaceous plants and incubated for 4-7days in moist chamber. Results demonstrated that the disease reduction was more evident in pre-treatment than in post-treatment, it was more remarkable especially on apple than pear and quince fruits The size of brown halo circle around the holes was restricted in pre-treatment. The color of fruits is not changed during incubation and disease symptoms were slowly developed during 1-week (Fig. 35., 36.).

Fig. 35. Reduction of fire blight disease by *Pantoea agglomerans* on fruits of pear from left: pre-treatment, post-treatment, and control

Fig. 36. Reduction of fire blight disease by *Pantoea agglomerans* on fruits of quince from left: pre-treatment, post-treatment and control

Flowers of *Cotoneaster* sp. were also treated with the same method as fruits in pre- and post- treatments and incubated for 5-days and recorded the disease symptoms caused by strain Ea29. The effect of *Pantoea agglomerans* in pre-treatment flowers was stronger than in post – treatment comparing to control, there was disease reduction in pre-treatment by the antagonist (Fig. 37.)

Fig. 37. Reduction of fire blight disease by *Pantoea agglomerans* on flowers of cotoneaster from left:, pre-treatment control and post--treatment

4.11. Disease reduction

Pantoea agglomerans demonstrated clear antagonistic effect on different pomaceous plants against many bacterial strains of *Erwinia amylovora* that caused fire blight and also was effective to *Xanthomonas vesicatoria* strains by *in vitro* experimental tests. The disease reduction achieved in pre-treatment was better than in post-treatment.

4.11.1. Reduction of bacterial spot disease caused by *Xanthomonas vesicatoria* in tomato plants treated with *Pantoea agglomerans*

Xanthomonas vesicatoria strain SO8 the causal agent of leaf spot disease on tomato was chosen for the *in vivo* application of the antagonistic strain HIP32, 24hr before (pre-treatment) and 24hr after (post-treatment) in green house experiments using cultivar 'Kecskeméti 262' with different developmental stages of tomato plants (6-12 leaves).

Disease symptoms were started 7-10 days after inoculations. Symptoms of the disease as small leaf spots more occupied the lower leaves than the upper ones. Determination of disease rating started in the same time when symptoms started to appear. Analysis of data was done according to multiple range analysis of variance.

Results demonstrated that on younger tomato plants *Pantoea agglomerans* was more effective and according to the data of disease rating it was recorded more effective in pre-treatment than in post-treatment (Table 22.).

The protective effect of pre-treatment was persisting during the whole time of experiments, but from 4th until 8th days the effect was nearly equal. There were no significance differences at 5% in the post-treatment effect during the whole experiment compared to control (as shown in appendix) but there was clear significance difference at 5% if compared to pre-treatment. Disease reduction was observed in all experiments in younger pre-treated plants (45%) more than in older pre-treated ones (30%). Disease reduction in post-treated younger plants was (24%) and older post-treated plants was (12%). Results gave an indication that *Pantoea agglomerans* when applied as pre-treatment have sufficient effects on tomato plants as well as it was on pomaceous plants (Fig. 38., 39., 40. and 41.) (Illustrated tables in appendix).

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Table 22. Disease rating in tomato plants treated by *Pantoea agglomerans* before (Pre-) and after (Post-) inoculation with *Xanthomonas vesicatoria* strain SO8

Advanced stage of seedlings	No. of plants / treatment	Days after symptoms appearance	Disease rating Pre-treatment	Control	Post-treatment
A*	6	1	1.00	1.50	1.20
B	12		1.20	2.30	2.00
C	13		1.99	2.33	2.08
D			2.01	2.50	2.33
E			2.33	2.92	2.50
A	6	2	2.00	3.10	2.30
B	12		2.10	2.33	2.20
C	13		2.10	2.90	2.90
D			3.70	4.60	4.0
A	6	3	2.00	3.10	2.36
B	12		2.25	3.20	3.00
C	13		2.95	4.30	3.83
A	6	4	2.20	3.38	3.00
B	12		3.00	3.40	3.33
C	13		3.10	4.20	4.00
A	6	5	2.50	3.62	3.15
B	12		3.00	4.0	3.60
C	13		3.00	3.83	3.50
A	6	6	2.70	3.83	3.83
B	12		3.00	3.83	3.50
A	6	7	3.20	5.00	4.50
B	12		3.00	4.00	3.65
A	6	8	3.33	4.70	4.70
B	12		3.00	3.40	3.33

Advanced stages (number of leaves) of tomato plants, A = 6 leaves; B=10, C,D and E = 12 leaves.

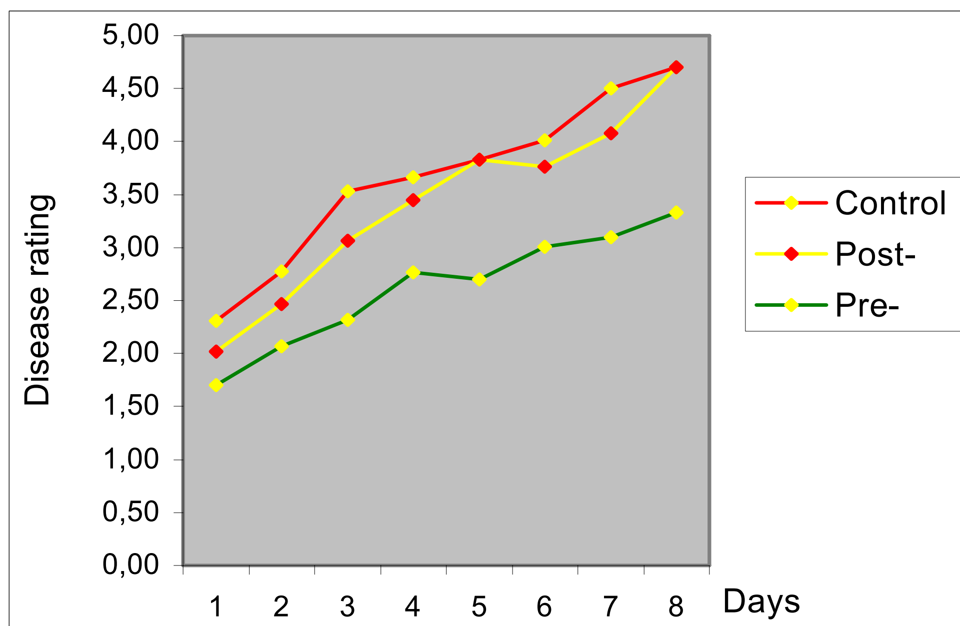

Fig. 38. Means of disease rating in tomato plants treated by *Pantoea agglomerans* before (Pre-treatment) and after (Post-treatment) with *Xanthomonas vesicatoria* strain SO8 (Pre- and post- are significantly difference at 5%) comparing to Control

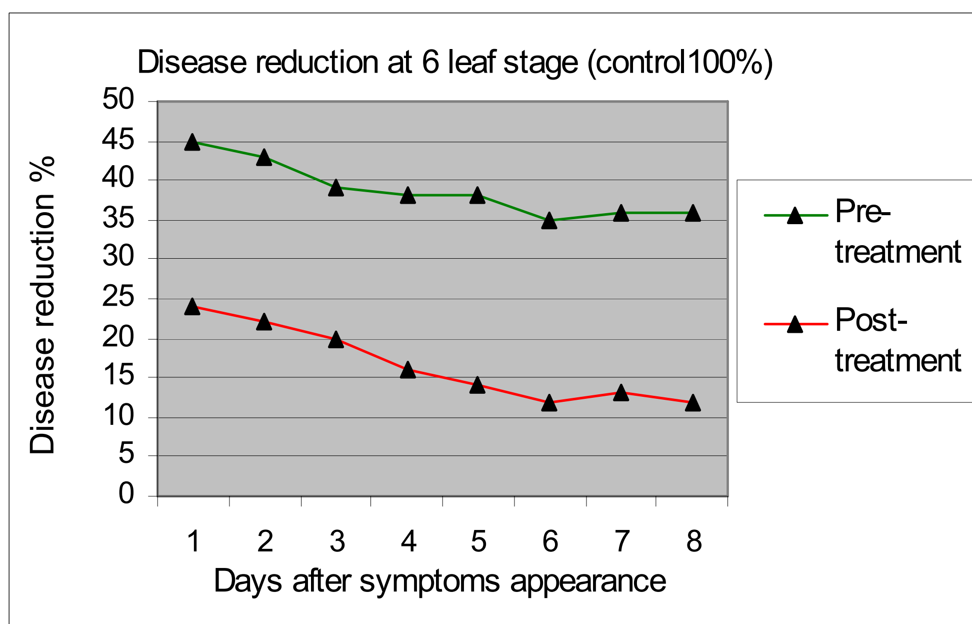

Fig. 39. Percentage of Leaf spot disease reduction pre- and post- treated by *Pantoea*

agglomerans **in 6 leaves tomato plants**

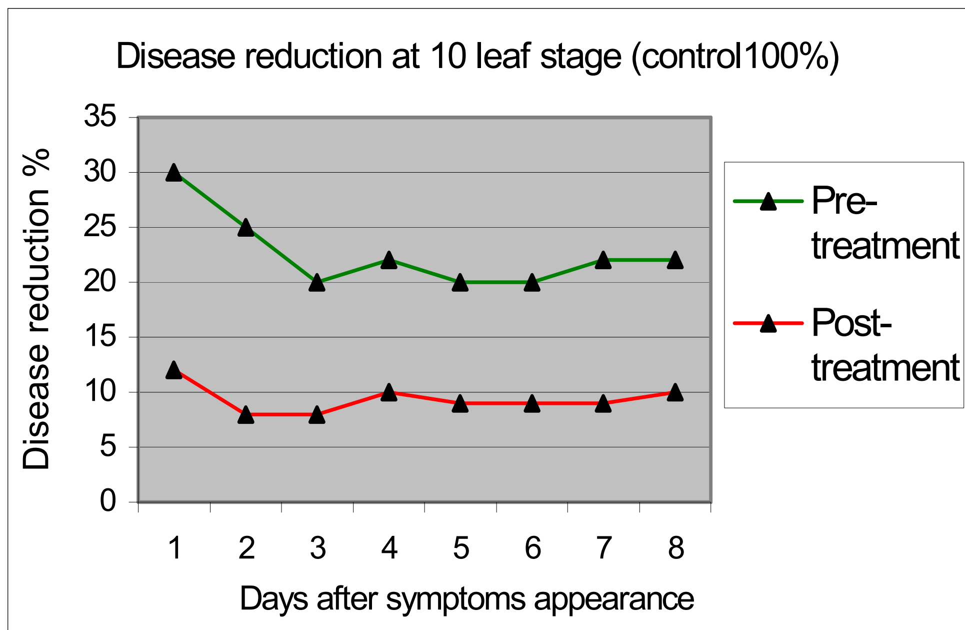

Fig. 40. Percentage of Leaf spot disease reduction pre- and post- treated by *Pantoea agglomerans* in 10 leaves tomato plants

Fig. 41. Disease reduction on tomato plants treated by strain HIP32 of *Pantoea agglomerans* before (A = Pre-treatment) and after (B = Post-treatment) inoculation with *Xanthomonas vesicatoria* (SO8) compared to control:(C)

Effectivity of strain HIP32 of *Pantoea agglomerans* was evaluated against leaf spot disease of tomato during different experimental times; December. – January (1998-1999), May – June (1999) and February. – March (2000). The disease rating was less in pre-treatment during May – June than in February. – March and December. – January treatments. According to calculations of disease rating the comparison of disease reduction in pre-treatment was at the average between 25 – 30% in Dec. Jan. and the average between 10 – 36% in Feb. – Mar. and average between 10 – 45% in May – June While in post-treatment the percentage of disease reduction was lower. (Fig. 42., 43., 44.)(Illustrated tables in appendix).

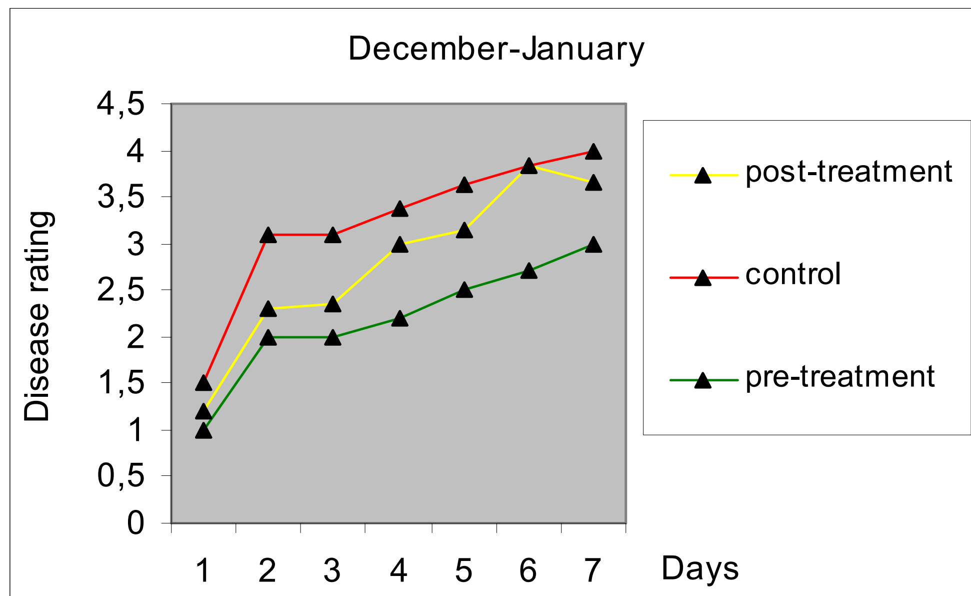

Fig. 40. Effect of *Pantoea agglomerans* on pre- and post-treated tomato plants inoculated with *Xanthomonas vesicatoria* (SO8) during December and January.

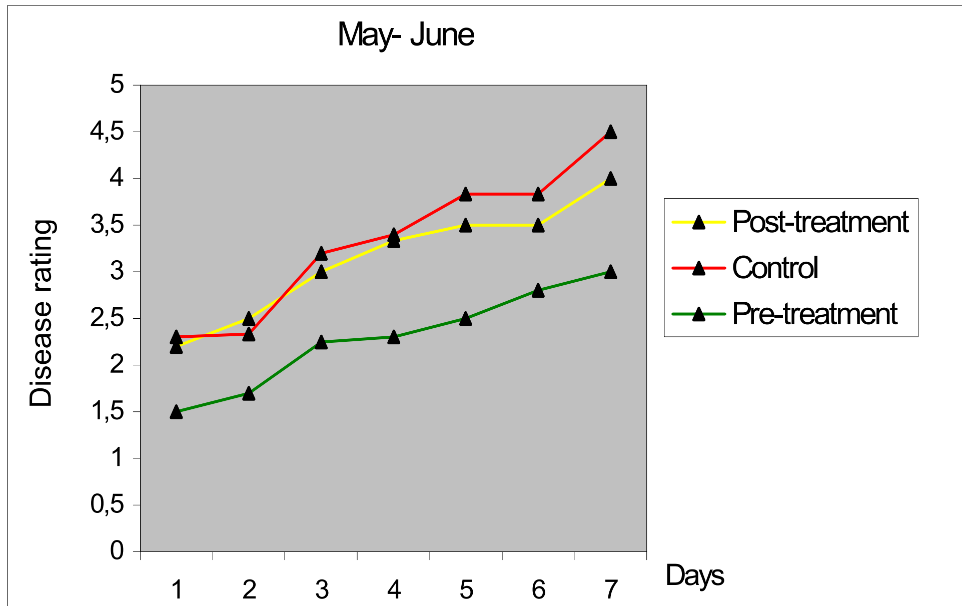

Fig. 41. Effect of *Pantoea agglomerans* on pre- and post-treated tomato plants inoculated with *Xanthomonas vesicatoria* (SO8) during May and June

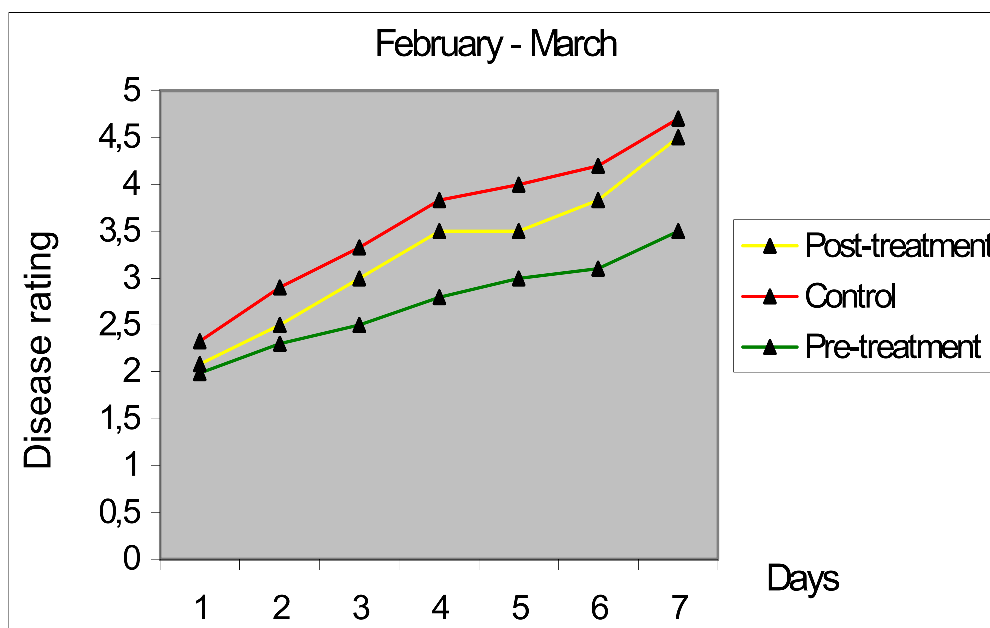

Fig. 42. Effect of *Pantoea agglomerans* on pre- and post-treated tomato plants inoculated with *Xanthomonas vesicatoria* (SO8) during February and March

4.11.2. Effects of *Pantoea agglomerans* on disease reduction caused by *Xanthomonas vesicatoria* in pepper plants

Pepper cultivar 'Cecei.SH' seedlings were treated in two independent experiments using 16 plants in each treatment. Bacterial suspension of strain HIP32 *of Pantoea agglomerans* was sprayed using the same method as in tomato and inoculated with strain Xv14 of *Xanthomonas vesicatoria*. Disease symptoms were recorded as small pale green - yellow scattered blisters or spots spreading to most of the leaves which later started to fall down. Evaluation of symptoms started 2 weeks after treatments by applying the disease index formula. Results demonstrated that the antagonistic effect of *Pantoea agglomerans* in disease reduction in pepper plants was more effective in pre-treatment (between 24 – 40%) than in post-treatment (9 – 30%) (Table 23. and Fig. 45. and 46.).

Table 23. Effect of *Pantoea agglomerans* on reduction of disease caused by *Xanthomonas vesicatoria* strain Xv14 in pepper plants

Disease rating				Disease reduction	
Days	**Controls**	**Pre-treatment**	**Post-treatment**	Disease reduction **% in Pre-treatment**	**Disease reduction % in Post-treatment**
1	2.3	1.2	1.7	40	30
2	2.6	1.4	1.9	38	27
3	2.8	1.8	2.1	36	25
4	2.9	2.2	2.5	24	14
5	3.5	2.6	3.2	26	9.0

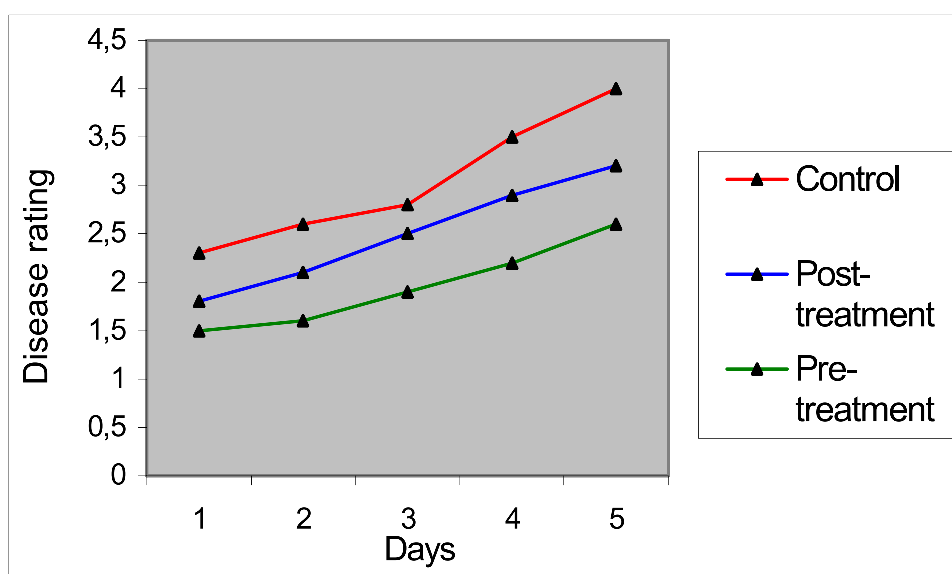

Fig. 45. Bacterial leaf spot disease rating in pre-treated and post-treated pepper plants with *Pantoea agglomerans*

Pre-treatment **Control**

Post-treatment **Control**

Fig. 46. Effect of *Pantoea agglomerans* on reduction of leaf spot disease caused by *Xanthomonas vesicatoria* strain Xv14 in pepper plants: (Pre--treatment to control), (Post-treatment to Control)

4.12. Effects of *Pantoea agglomerans* on pepper plants development and to disease reduction caused by *Xanthomonas vesicatoria*

Bacterial spot disease was tested in pepper seedlings to evaluate the effect of *P. agglomerans* on disease reduction. *Pantoea agglomerans* strain HIP32 was applied 24hr before and after inoculation with *Xanthomonas vesicatoria* (Xv14*)*. An additional effect of *Pantoea agglomerans* was observed in connection with leaf-size and numbers in treated plants. Results were analyzed using multiple range analysis and one way analysis of variance, which demonstrated that before, and after inoculation (pre-treatment and post-treatment) had almost the same effect on growth of pepper plants when leaf size and numbers were counted started 4-weeks after treatments. Leaf size was larger and also leaf numbers were more than in the controls where leaves were smaller in size and early falling. (Table 24. and Fig. 47.).

<u>Table 24.</u> Influence of *Pantoea agglomerans* on average of leaf size and numbers in pepper plants during reduction of disease caused by *Xanthomonas vesicatoria*

Average of leaf area (mm) / leaf number / treatment					
Leaf number	***Control***	**Leaf number**	**Pre-treatment**	**Leaf number**	**Post-treatment**
1	7.50	4	7.81	3	5.00
2	1.63	5	3.75	4	4.94
2	3.63	5	6.10	4	10.63
3	2.08	5	7.50	5	3.35
3	2.17	6	3.63	5	5.45
3	3.08	6	8.67	6	4.50
3	3.25	6	5.42	6	5.58
3	5.75	6	5.75	6	6.79
3	3.92	7	5.82	6	10.63
3	7.00	7	9.54	7	4.64
4	4.00	7	10.57	7	9.32
4	4.75	8	9.00	7	12.11
4	12.31	8	10.25	8	4.84
5	5.10	8	11.91	8	9.31
5	6.75	8	14.00	9	14.39
5	10.65	8	14.06	9	21.11

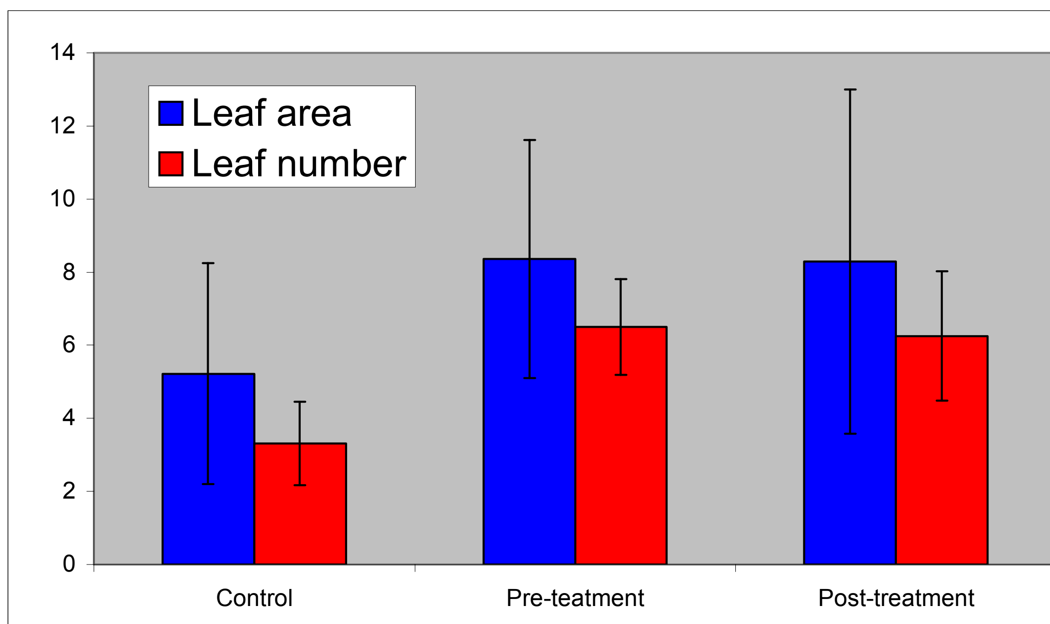

Fig. 47. Influence of *Pantoea agglomerans* on leaf growth (expansion of leaf area) and on number of pepper leaves inoculated with *Xanthomonas vesicatoria* (Xv14)

4.13. Length of protective effect in pepper plants achieved by *Pantoea agglomerans* against damage caused by *Xanthomonas vesicatoria strain Xv14*

Pepper plants previously treated with antagonistic strain HIP32 of *Pantoea agglomerans* were inoculated with *Xanthomonas vesicatoria* strain Xv14 through 1week in daily treatments (started from 1st to 7th days). Disease severity on pepper leaves was compared to control during 7 days, beside reduction in disease severity the persistence of protective effect of *Pantoea agglomerans* was found more suitable at first and 4th day after pathogen treatment. Other treatments were also effective compared to the control, but had less effect compared to the first day treatment (Table 25. and Fig. 48. and 49.).

Table 25. **Persistence of antagonistic effectivity of *Pantoea agglomerans* in pepper seedlings inoculated with *Xanthomonas vesicatoria* strain Xv14 at different times (1-6days) after antagonistic pre-treatment**

Disease category (of leaf spots)	Number of leaves				
	1.day	**2.day**	**4.day**	**6.day**	**Control**
1 – 10	39	43	34	27	29
11 – 25	22	14	20	20	30
26 – 50	15	24	33	12	27
51 – 75	32	48	40	24	56
76 – 100	35	85	95	120	150
∑ of leaf spots/ leaf area	**143**	**214**	**222**	**203**	**292**
Means	**2.01**	**2.03**	**2.07**	**2.95**	**3.75**

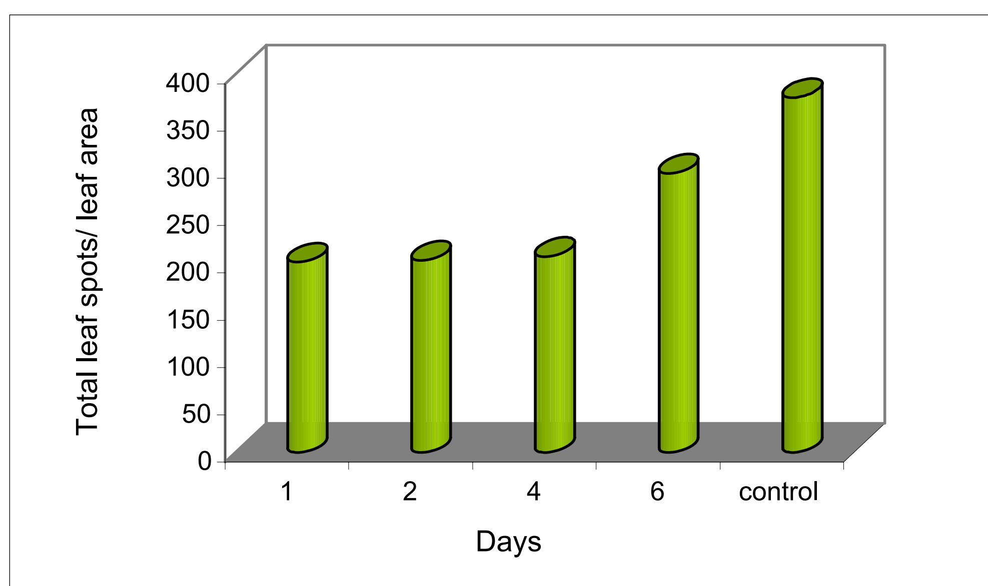

Fig. 48. Length of persistence of protective effect of *Pantoea agglomerans* in pepper plants inoculated with *Xanthomonas vesicatoria* strain *Xv14*

The protective effects of *Pantoea agglomerans* on pepper plants was starting 24 hours after application of *Xanthomonas vesicatoria* strain Xv14 as (pre-treatment). The length of duration of the effect had extended to few days after (4 - 6 days) but the optimal effect had lasted for 4days.

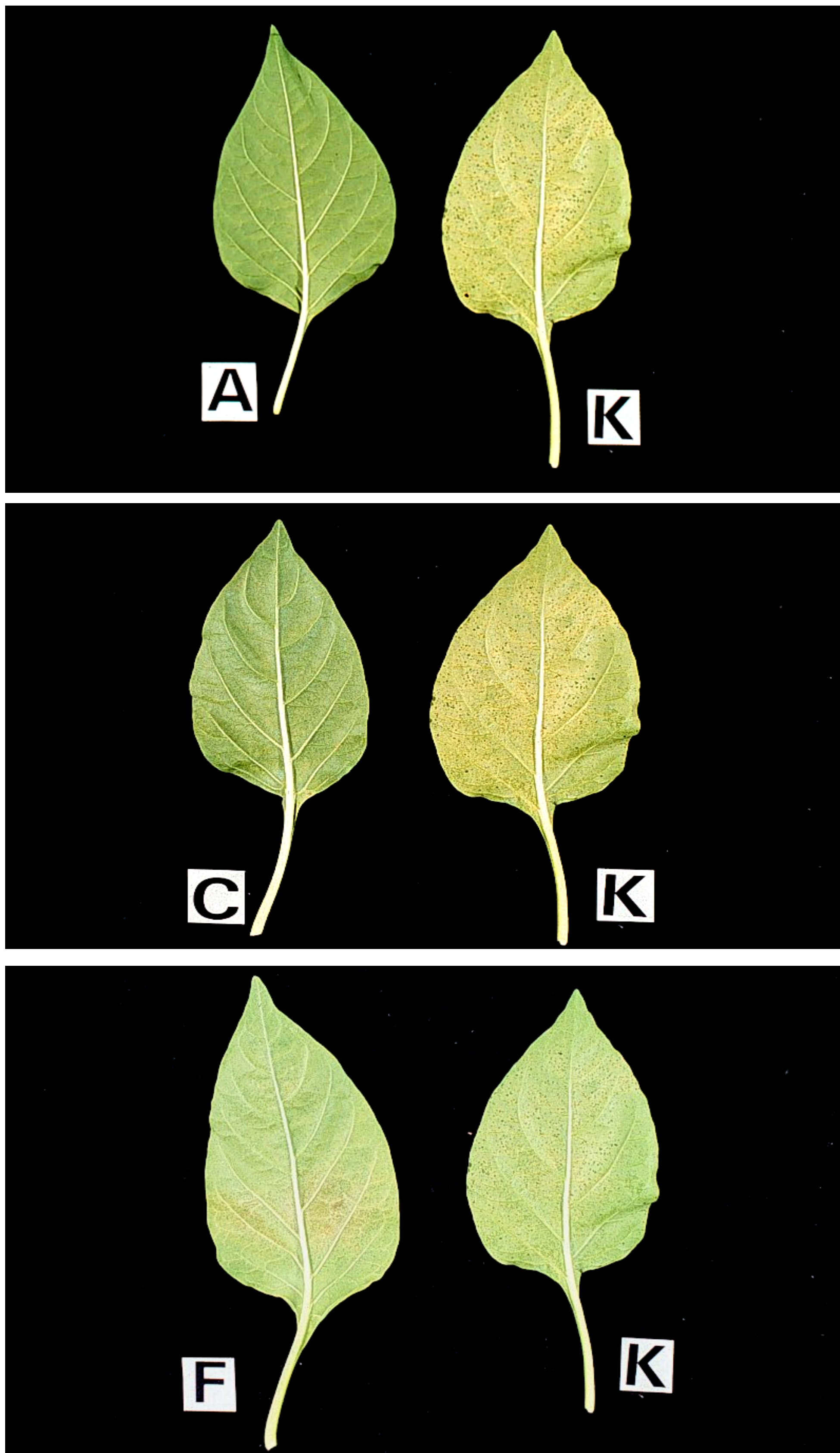

Fig. 49. Persistence of antagonistic effectivity of *Pantoea agglomerans* on pepper seedlings inoculated with *Xanthomonas vesicatoria* strain Xv14 at different times 1, 4, 6 days as A, C, and F: (1 day = A; 4 days = C; 6 days = F; and K = control)

5. Discussion

Bacteria multiplying inside the intercellular spaces of tissues can hardly be reached with chemicals or antibacterial substances, which have been used until now and caused hazard to human beings, animals, plants and the environment. When the disease occurs in clusters, overcoming it is in most cases unsuccessful.

Therefore, we must not depend entirely on defensive substances but preventive measures should be taken first so that the pathogen can be kept far from the plant either by quarantine regulations or using healthy seeds. A considerable advantages of using antibiotics as opposed to other plant protective agents is that they have a good absorbing ability and they translocate in tissues which is considered significant with bacterial diseases.

Streptomycin sulfate was used as an antibiotic against bacterial plant pathogens especially against fire blight disease in pear and apple (Ark and Scott,1954) and it was proven to be an effective bactericide with no phytotoxic effects at the recommended doses. However, its use in agriculture has been prohibited in many countries, because of development of resistance to streptomycin since 1971 not only by *Erwinia amylovora* but also by other microorganisms in the plants or in the soil or water, including possible human and veterinary pathogens (Jones and Schnabel, 2000).

Streptomycin sulfate, Kasugamycin and other antibiotics are now in use in many countries for the control of several bacterial plant diseases but the utilization of streptomycin sulfate in other countries is fasten to the permission of higher authorities from time to time in each case. The most suitable schedule is a weekly spraying regime, which is quite expensive. When spraying is neglected for longer than 10 days, successful results cannot be insured. However, no complete protection can be expected by chemical neither with antibiotics treatment alone (Vanneste, 2000). In Hungary streptomycin sulfate can be used only on the occurrence of fire blight, with strict regulations.

Over more than 30 years, biological control has advanced from a subject of basic research to a feasible component of an integrated disease management program. This worldwide advance has been propelled by selection of effective antagonistic strains, by enhanced knowledge of the mechanisms by which these strains suppress disease and increased understanding of the ecology of bacterial epiphytes on plant surfaces. Moreover, the growers have begun to accept biological control as a complementary strategy, which can be used effectively with other forms of disease suppression.

However, because no complete protection can be expected by chemical treatment alone, appearance of resistance e.g. in *Erwinia amylovora* to streptomycin have accentuated the need for alternative control strategies. To be viable, these strategies need to mesh with consumer and food safety standards which in many countries, have created a marketing and regulatory climate that requires growers to produce high-quality fruit with minimal residues of synthetic chemicals (Vanneste, 2000).

Biological control on aerial plant surfaces is much less well-developed than in the soil / rhizoplane for two main reasons (a) antagonists can maintain themselves more readily in the soil because of the more uniform environment, and (b) the availability of cheap and effective pesticides and their ease of application to plant foliage has discouraged the use of biological control on aerial surfaces.

Further research is therefore needed to develop suitable antagonists that can be effective in the foliar environment. It is unlikely that a perfect antagonist for the control of a particular pathogen will be found in nature. However, it may be possible to improve an organism's disease control capabilities by selection for those characteristics that would lead to greater effectiveness (Blakeman and Fokkema,1982). Biological control in Hungary is limited to the conservation of natural antagonistic flora and fauna. Also antagonistic effects of many microorganisms including PGPR against many pathogens are under experimental tests (Biró et al.,1998, 1999) and to the application of imported biopesticides and chemical plant protection products such as Koni ((Dormanns-Simon, 1993, Dormanns-Simon et al., 1997)

The purpose of this study has been to investigate the possible utility of characterized antagonistic strains from the microflora of phylloplane of apple trees) and also from rhizosphere of soil samples collected from Libya and Hungary against diseases which are considered economically serious such as fire blight of pomaceous trees caused by *Erwinia amylovora*, bacterial spot disease caused by *Xanthomonas vesicatoria* in pepper and tomato and bacterial canker of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis.*

It was found in the apple microflora numerous bacterial species with different colony types, shape, and color also had different antagonistic effects against tested plant pathogenic bacteria that belong to different genera. To confirm the effectivity of the antagonists different bacterial and fungal indicator pathogens were used in antagonistic tests. Inhibition zones measurements demonstrated that some antagonistic isolates displayed wide spectrum and strong effectivity against these pathogens.

These results were in accordance with results of Blakeman and Fokkema (1982) Andrews (1985) and Campbell (1989) in a screening of biological control agents from phylloplane against plant pathogens. Results by Zeller and Wolf (1996) and Vanneste (1996) confirmed the utility of epiphytic bacteria of apple trees against the fire blight pathogen.

According to the data, the antagonistic isolate HIP32 was chosen for further studies because it exhibited different levels of antagonistic activity against all plant pathogenic species tested (as clarified by strong inhibition zones measured in millimeters), also showed antagonistic effects on some fungal isolates such as *Fusarium oxysporum* isolates and *Geotrichum candidum* as well. It had wide antimicrobial spectra. It is in accordance with the results of (Filippi et al., 1984, Yuen et al, 1985, and Bochow, 1989). This isolate showed pronounced effects on most strains of fire blight disease caused by *Erwinia amylovora* in pomaceous plants and the bacterial leaf spot disease caused by *Xanthomonas vesicatoria* (in pepper and tomato isolates).

Methods used to determine the antagonistic effects were not enough to clarify whether inhibition is in connection with the change of pH or it is a result of production of metabolites of *Pantoea agglomerans.* During cultivation of *P. agglomerans* in puffered Ayre's basal or PDB media the optimum pH was 7.0 and after 7 days of cultivation only a slight change in pH was recorded (6.5 - 7.2).

The antagonist displayed poor growth below pH 6. A bacterial growth of pathogenic strains of *Xanthomonas vesicatoria* and *Erwinia amylovora* could tolerate lower pH about 5. Therefore, no change in pH of the media during the in vitro experiments. It can be concluded that the antibacterial effects of strain 32 were due to the metabolite production and not to pH changes in the medium, which had a role in an inhibition zone formation.

The antagonistic effect was found to be due to the role of metabolites of the antagonist in the medium, it was clarified by other ways too. The culture filtrate (CF) of strain HIP32 of *P. agglomerans* was prepared several times (applying potato dextrose broth PDB and Ayre's basal media) against *Erwinia amylovora* strains Ea1, Ea23 and *Xanthomonas vesicatoria* strain Xv14 and *Clavibacter michiganensis* subsp. *michiganensis* strain Cm3.

Results demonstrated the presence of metabolites in the culture filtrate of antagonistic strain HIP32 causing inhibition zones around the CF holes. The culture filtrates as well as the bacterial cells of strain HIP32 were effective against plant pathogenic bacterial strains. Ayre's synthetic basal medium was found more suitable for metabolic activity of strain HIP32 than potato dextrose broth medium.

Activity of the culture filtrate was realized by mixing the antibacterial metabolites (CF) of strain HIP32 with different dilution of PDA medium before inoculation with the pathogen. Reduced numbers of colony forming units of the tested pathogenic strains were observed. This indicated that during its multiplication in Ayre's medium the antagonistic strain HIP32 produced metabolites in culture, which have an effect not only on the number of colony forming units (CFU) but in a certain dilution also affect colony size of the pathogenic strains. More concentrated culture filtrate had more effect on CFU of the pathogen but no resistant colonies were observed.

Production of metabolites by antagonistic bacteria was reported by many authors (Thiry-Braipson et al., 1982, Ishimaru et al., 1988, Wodzinski et al., 1987, Wodzinski and Paulin, 1994, Jabrane et al., 1996, Amellal et al., 1998 and Vanneste, 2000) who demonstrated and clarified metabolite production by antagonistic strains from *Enterobacteriaceae*, especially *Erwinia herbicola* (synonym of *Pantoea agglomerans)*, which was effective against many plant pathogenic bacteria including the fire blight pathogen, *Erwinia amylovora*.

Metabolite production of strain HIP32 of *Pantoea agglomerans* was confirmed by a time dependence test too. It was studied in order to investigate the effect of time by cultivating bacteria at different intervals. According to data of inhibition zones (in mm), strains *Ea*1 of *Erwinia amylovora* and Xv14 of *Xanthomonas vesicatoria* were more affected by metabolite activity of the 6th and 8th days than Ea17 and SO8 and than cultivation at shorter times. In fact testing the quantity and the quality of the metabolites is needed and it was not possible by the restrict of time of the study.

These results were encouraging to start both *in vitro* and *in vivo* preliminary tests to clarify the antagonistic effect of the selected antagonistic isolate HIP32. We could identify isolate HIP32 using different methods of characterization and determined it's taxonomic position first by confirmation of its nonpathogenic or saprophytic ability through pathogenicity tests in pepper, tomato, tobacco plants and apple which clarified that it couldn't cause any damage in these tested plants which means it is a saprophytic.

Isolate HIP32 was unable to induce the hypersensitive reaction in tobacco leaves by applying HR technique (Klement et al.,1964). Saprophytic bacteria are unable to induce HR in tobacco or other hosts, they cannot produce neither typical disease symptoms nor necrosis as part of a hypersensitive reaction (Klement et al.,1990). Isolate 32 was also unable to produce pectolytic enzymes or display any pectolytic activity in potato slices confirming its saprophytic ability.

These results are in accordance with (Lelliott and Dickey, 1984, Klement et al., 1990). The results of cultural and biochemical characterization showed that strain 32 belongs to family *Enterobacteriaceae*. Other biochemical tests (H_2S, phenylalanine deaminase, and nitrate reduction) confirmed that strain HIP32 belongs to the *Enterobacter spp*. Confirmation of these results was found in works of other authors; (Brenner, 1984).

Additional biochemical tests to separate species of *Enterobacter* from one another were applied including microtests of API 20 E, which confirmed that our isolate HIP32 is *Pantoea agglomerans* which is the current accepted name as proposed by Gavini et al., (1989). These results were concordant with those of Ewing and Fife, (1972), Leete, (1977), and Brenner, (1984).

There are more than 35 synonyms for the organisms grouped as *Enterobacter agglomerans*. Other names were used for *Pantoea agglomerans* the synonym of *Enterobacter agglomerans* such as *Erwinia herbicola - Erwinia milletiae* complex on the basis of fatty acid profiles and DNA homology. Most of these names were given to strains isolated from various plant sources or soil. (Dye, 1969, Leete,1977, Gavini et al, 1983, Brenner et al., 1983). Recently, the synonym *Pantoea agglomerans* comb. Nov. is used together with *Enterobacter agglomerans* on the basis of DNA-DNA hybridization and polyacrylamide gel electrophoresis of soluble proteins (Gavini et al.,1989).

From the biochemical characteristics of strain HIP32 and from the main characteristics that differentiating its biogroups such as production of gas from glucose, negative for Arginine (ADH), Ornthine (ODC), Lysine (LDC) and Indole but positive for Voges-proskauer and Sorbitol tests it was found that *Pantoea agglomerans* strain HIP32 belongs to bio-group G1 of aerogenic strains which have confirmed the results of Ewing and Fife (1972). These strains were isolated from plants, flowers, seeds, vegetables, water, soil and foodstuffs and chiefly charactrerized by the absence of LDC, ODC and ADH and by the synthesis of nondiffusible yellow pigments. Ewing and Fife (1972) and Brenner et al., (1983) showed that some strains of *Enterobacter* species orginated from human of other biogroups are considered opportunistic pathogens causing nosocomial secondary infections.

Considering the possible utilization of *Pantoea agglomerans* as a biocontrol agent in the field of plant protection: Different tests were carried out to study the persistence and adaptation of strain HIP32 to its original host which was necessary to clarify the seasonal viability of its population. Establishment and multiplication of *Pantoea agglomerans* in apple trees was found to be successful (few weeks of sampling at three different seasonal times). Re-isolation were made using selective medium (supplemented with Trimethoprim which was chosen due to

antibiotic sensitivity tests, which clarified the resistance of strain HIP32 to this antibiotic and kill other microorganisms). Metabolic activity of re-isolated strain HIP32 was proved periodically against different pathogenic strains.

Determination of the colony forming units of the population of strain HIP32 indicated that during one-month period it was not only present in high level in apple leaves but also an increase in its multiplication was recorded. The same results however, were proved by Kearn and Hale (1993) that *Erwinia herbicola (*the synonym of *Pantoea agglomerans)* was isolated from apple flowers after 4 days in 10 – 40% of sprayed initial population, but after 10 days the population has increased 400-800 times as compared to the natural epiphytic population of the bacteria. Kearn and Hale (1995) indicated the incidence of these inhibitory bacteria in blossoms infected with the fire blight pathogen in apple orchards.

These results encourage the work for *in vivo* application of such biocontrol agents because *Erwinia amylovora* usually infecting the host trees during blossoming in that time biocontrol agent could be active when the application of chemicals is not possible but only that of antibiotics with frequent applications. The main problem with bacteria as biocontrol agents is their ability to survive on plant surfaces in natural conditions.

On leaf surfaces of apple trees many epiphytic pathogens as well as saprophytic bacterial species can grow. These bacteria can penetrate the leaf stomata or natural openings and can initiate the disease inside the intercellular spaces of leaf tissues. It can be concluded that strain HIP32 of *Pantoea agglomerans* predominate among microorganisms of the aerial surfaces of pomaceous plants. This species establishes and develops a large population on the stigmatic surface prior to the establishment of *Erwinia amylovora.* Opinion of other authors is that through a combination of mechanisms these antagonistic populations suppress the establishment and epiphytic growth of the pathogen.

Suppression of growth of *Erwinia amylovora* population on stigmatic surfaces reduces the probability of floral infection and spread of the pathogen to other blossoms (Wilson et al.,1992, Wilson and Lindow, 1993, Johnson et al.,1993). Fire blight of pomaceous plants is a good candidate for biological control because bacterial antagonists need to persist on nutrient rich stigmatic surfaces for only about a week in order to suppress blossom infection effectively (Vanneste, 2000).

The opinion in this study that the less effectivity of the antagonist in post-treatment is that the pathogen pre-colonized the intercellular spaces 24 hr before the antagonistic strain application which could be difficult for antagonist to overcome easily by any of its mechanisms

of suppression. The pre-presence of antagonistic bacterial species could suppress the activity of the pathogens.

Previous *in vitro* tests showing successful results of the pre-application of strain HIP32 of *Pantoea agglomerans* against many strains of *Erwinia amylovora* encouraged us to continue the experiments with plants. Quarantine measures also prohibit field testing by *Erwinia amylovora* so we had to use *in vitro* laboratory methods using different cultivars of apple trees. By applying the antagonistic strains in different mode of actions as pre- or post–treated - plant parts to apply the possible active mode in next tests.

Developed methods with leaf discs, complete leaf, fruit and flower of pomaceous plants were used. It was concluded that strain HIP32 of *Pantoea agglomerans* is able to cause a reduction of fire blight disease in some apple cultivars as assayed by leaf disc method. This effect was selective and not uniform, in some cultivars strain HIP32 showed pre-treatment to be more effective while in others the biocontrol effect was nearly the same in post-treatment as in pre-treatments.

After obtaining good results in leaf discs of apple cultivars we started to continue other tests following the epiphytic stage of *Erwinia amylovora* with different pomaceous species. Leaves of apple, pear, quince, and cotoneaster were selected for applying the antagonistic strain HIP32 in pre-treatment and post-treatment with antagonistic strain HIP32 and inoculated with a suspension of their corresponding strain of *Erwinia amylovora (*Ea1, Ea17, Ea23, and Ea29).

It can be concluded that the disease reduction in pre-treatment was more evident than in post-treatment especially on quince and cotoneaster leaves. Results of treated fruits demonstrated that disease reduction by *Pantoea agglomerans* in quince was more significant than in apple and pear fruits and the effectivity was more or the same as in pre-treated as in post-treated fruits of apple, pear as well as in flowers of cotoneaster.

Similar results were demonstrated by Klos (1963), Beer and Rundle (1983) Beer et al, (1984) and Vanneste (1996) who proved that *Erwinia herbicola* strains could cause disease reduction on apple and pear blossoms infected by *Erwinia amylovora.* During pre-treatment, the antagonist colonized the intercellular spaces first before the application of the pathogen, which gave a chance for the antagonist to multiply and produce its metabolites.

However, it is probably difficult for the antagonist to compete with the pathogen in post-treatment because the pathogen starts to grow and multiply in favorable conditions of humidity and temperature inside the intercellular spaces.

Successful results obtained by *in vitro* tests of *Pantoea agglomerans* against different plant pathogenic species, especially *Xanthomonas vesicatoria* strains and the results of tested plant parts lead to continuation of the *in vivo* antagonistic experimental studies. In greenhouse conditions using other plant-pathogen combinations disease reduction of bacterial spot disease in tomato and pepper plants caused by *Xanthomonas vesicatoria* was demonstrated.

Disease reduction was investigated in treated tomato and pepper plants by the antagonistic strain *Pantoea agglomerans.* 24hr later treated plants were inoculated with different strains of *Xanthomonas vesicatoria* as pre-treatment and by reverse the order of inoculation in post-treatment. Statistical analysis of the visual recording of symptoms showed more disease reduction in pre-treated plants than post-treated one. The antagonist had an effect on tomato and pepper plants as seen by their vigour growth, reduced number of spots, and reduced yellowing, which can be considered as a good *in vivo* effect of the antagonist. These achievements in the results on pepper and tomato are the first to clarify the effect of *Pantoea agglomerans* on strains of *Xanthomonas vesicatoria*

The disease reduction was achieved and repeated in different developmental stages of tomato seedlings especially in younger than in older ones. Results showed that pre-treatment was always more effective. The greenhouse conditions such as temperature, soil conditions, humidity, light and fertilizers have some effect on multiplication of the antagonists as well as on the indicator pathogens, which could cause the stimulating of the effectivity, these factors were not studied. Disease symptoms were calculated by different methods of statistical analysis using disease-rating categories.

In the above tests the results confirmed the effectivity of the epiphytic strain HIP32 on different host plants including pepper plants and that the pre-treatment was always has the strongest effect.

The leaf size and numbers of pepper leaves were statistically calculated in pre- and post-treated plants. Beside the direct effect on disease reduction (reduced leaf spot numbers and leaf fall), more vigor, strong green treated plants were observed. There were also additional effects of the biocontrol agent on improvements in leaf size (expansion) and increasing leaf numbers, which may have economical value.

Persistence of the protective effect of *Pantoea agglomerans* in pepper plants demonstrated that it is suitable to apply antagonistic isolate as a suspension by spraying 24 hr before pathogen inoculations. Results of disease symptom reduction was demonstrated that the protective effect was evident from first day until 6th days of treatments and confirmed our previous results that pre-treatment was more effective in reducing the disease symptoms during the period (1 – 7 days).

It can be conclude that during pre-treatment, the antagonist colonized the intercellular spaces first before the application of the pathogen, which gave a chance for the antagonist to multiply and produce its metabolites. However, it is probably difficult for the antagonist to compete with the pathogen in post-treatment because the pathogen starts to grow and multiply in favorable conditions of humidity and temperature inside the intercellular spaces.

After application of the isolated strain HIP32 from phylloplane on many host plant species against some important diseases in different *in vitro* and *in vivo* tests, the soil rhizosphere was also interesting subject by starting preliminary isolation and selection of soil antagonists against the tested pathogenic species. Rhizosphere soil samples were screened *in vitro* for selection of soil antagonists against most plant pathogenic bacteria and fungi used in this study by the double layer technique and streaking method. Libyan soils have different alkalinity levels but rarly acidic, samples were ranged from fallow - sandy clay to sandy loam and sandy. Hungarian soils have different pH levels but many are acidic, samples were ranged from fallow clay loam to fallow clay or sandy clay.

The measurement of inhibition zones of many antagonists against *Clavibacter michiganensis* subsp. *michiganensis* and *Agrobacterium vitis* indicated that there are many bacterial antagonists showed selective effects to these pathogens can be isolated from soil. The richest soil with antagonists in Hungary was from Zsámbék (1) (sandy clay) while in Libya the richest was Benghazi (2) (sandy-clay) too. The following authors are in accordance with these results about the possibility of isolation of antagonists from the soil rhizosphere (some of these isolates exhibited a pronounced and a wide spectrum antagonistic effects as determined by inhibition zones) (Farrago et al., (1980), Kerr (1980), Weller (1988), Keel (1992) and Haansuu et al., (1998).

One isolate coded as (HIR225) have been chosen for further studies because of its wide spectrum effects against most bacterial plant pathogens and of its strong inhibition effects against most species tested.

Soil isolate HIR225 could be identified using different methods of characterization and determined its taxonomic position first by confirmation of its saprophytic ability through pathogenicity tests on pepper, tomato, tobacco and apple which clarified that it couldn't cause any damage on these tested plants.

The soil isolate was unable to induce the hypersensitive reaction (HR) in tobacco leaves. Saprophytic bacteria are unable to induce HR in tobacco or other hosts, isolate HIR225 was also unable to produce pectolytic enzymes in potato slices confirming its saprophytic ability. The

results of cultural and biochemical characterization such as motility, spore forming ability, KOH reaction have shown that strain HIR225 belongs to the family *Bacillaceae*

For determination of species level of genus *Bacillus* we have used numerous biochemical tests, which confirmed that strain HIR225 belongs to *Bacillus subtilis.* Confirmation of these results were found by other authors in Barrow and Feltham (1993) and Kerbs et al.(1998).

For preliminary utilization of soil strain HIR225 as biocontrol agent against diseases also for determine whether the phylloplane strain HIP32 can grow or being active in soil or not (because *Pantoea agglomerans* is a soil habitant). The population of (*Clavibacter michiganensis* subsp. *michiganensis* strain Cm3 and *Xanthomonas vesicatoria* strain SO8) were followed by re-isolation of their colony forming units from the soil after mixed with the antagonists (*Pantoea agglomerans* and *Bacillus subtilis*) during 4 weeks in different independent experiments. A selective media have prepared using nitrofurantoin to re-isolate strains (Xv14, SO8) of *X. vesicatoria* and lincomycin to re-isolate strain Cm3 of *Clavibacter michiganensis* subsp. *michiganensis* in comparison to antagonistic strains (HIP32, HIR225) which were inhibited by these antibiotics.

Similar results were proved by Ericsson and Sherris (1971) who used nitrofurantoin to inhibit *Enterobacter agglomerans,* also lincomycin have better effect on soil microorganisms as well as *Clavibacter michiganensis* subsp. *michiganensis* when supplemented in selective medium (Ujfalussy, 2000). It can be conclude that these two antibiotics were successful at 100ppm for application in the quantitative analysis of survival of the pathogens in soil under the influence of the above antagonists.

The fallow sandy soil type (Őrbottyán) was also tested against the above antibiotics and results showed that these antibiotics have a good effect for the soil natural microflora. Soil experiments conducted by using the epiphytic *Pantoea agglomerans* and the soil *Bacillus subtilis* strains against *Xanthomonas vesicatoria* strain SO8 and *Clavibacter michiganensis* subsp. *michiganensis* strain Cm3

Re-isolation of pathogens was made on selective media that were prepared for both pathogens. Weekly samplings were made for each soil sample It was concluded that the number of colony forming units of the pathogens were reduced in accordance with time as a result of antagonistic activity of strains *Pantoea agglomerans* and *Bacillus subtilis* in the soil as compared to the control. Similar results have been demonstrated by other authors (Dupler and Baker, 1984, Amellal et al., 1998).

Ujfalussy (2000) found that resistant colonies of *Bacillus subtilis* strain FZB38 to antibiotic action were formed after few days in the culture zone, but in our strain no resistant colonies were formed even after few weeks. These results explain that strain HIR225 of *Bacillus subtilis* is different because it was isolated from Libyan soils which gave an idea to use it as biological control agent tolerating the Libyan weather and soil conditions. This strain which have tested to a given Hungarian soil type hoping that it is better than other strains of *Bacillus subtilis* reported in the literature.

The work should be continued on both antagonists *Bacillus subtilis* and *Pantoea agglomerans* afterwards in Hungary or in Libya where should be tested for adaptation to *in vivo* adverse conditions e.g. the tolerance of these strains to different climatic conditions and other plant host-pathogen combinations

Hoping that in this study I have selected some important biocontrol agents and my work will be a model for additional experiments in future on selecting other candidate biocontrol agents from naturally occurring epiphytic bacteria in leaves of different economically important crops. On the long run the prespective of these biocontrol agents can provide a protection to nature from the overuse of toxic chemicals that threatens human life.

SUMMARY

Screening for bacterial antagonistic strains of apple trees phylloplane and of soil rhizosphere samples was conducted against most economically important diseases such as fire blight of pomaceous crops caused by *Erwinia amylovora*, bacterial spot of pepper and tomato caused by *Xanthomonas vesicatoria* and bacterial canker of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis.* Numerous isolates were found to be antagonistic to most plant pathogenic bacterial strains tested. Some of these antagonists showed wide spectra and clear antagonistic effects by inhibition zones.

Two isolates were chosen for further studies, isolate HIP32 from the phylloplane and isolate HIR225 from the rhizosphere. They showed clear inhibition effects to most pathogensic strains tested and nonpathogenic characters on test plants.

Taxonomic position of the isolates was confirmed. Isolate HIP32 was *Pantoea agglomerans* aerogenic biotype G1 and isolate HIR225 was *Bacillus subtilis*.

During *in vitro* tests of antibacterial activity of *Pantoea agglomerans* effect of metabolite production on indicator pathogens was determined. For utilization of *Pantoea agglomerans* as a biocontrol agent establishment or persistence of the antagonisti in the original host apple trees was accomplished in field conditions. Multiplication was clarified during summer season.

The populations of *Xanthomonas vesicatoria* and C*lavibacter michiganensis* subsp. *michiganensis* were followed. *Bacillus subtilis and Pantoea agglomerans* had a significant biocontrol effect on reducing bacterial cell numbers of the pathogens in the treated soil.

Disease reduction of fire blight using *Pantoea agglomerans* in organs of some pomaceous plant species (apple, pear, quince, and cotoneaster) was confirmed. Effectivity of *Pantoea agglomerans* in reduction of bacterial leaf spot disease was demonstrated. The antagonist gave noticeable effects (about50%) when applied before the pathogen inoculation.

Pantoea agglomerans not only reduced the number of necrotic leaf spots but also reduced the leaf fall. Treated plants with the antagonist had improved good bearing and vigour.

In this study *Pantoea agglomerans* had capability to reduce disease symptoms and pathogen cell numbers, could be prospective as biocontrol agent. *Bacillus subtilis* could reduce the bacterial cell numbers in soil, so it could be proposed as biocontrol agent in contaminated soils.

Összefoglalás

Antagonista baktériumfajokat izoláltunk almafilloplánból és talajmintákból, melyek hatásosak voltak néhány gazdaságilag jelentős baktériumos betegség leküzdésére, így az almatermésű növények tűzelhalásával szemben, melyet az *Erwinia amylovora* okoz, a paprika és paradicsom baktériumos varasfoltosság betegségének leküzdésére, melynek kórokozója a *Xanthomonas vesicatoria*, a paradicsom baktériumos rákjának kórokozója, a *Clavibacter michiganensis* subsp. *michiganensis* ellen. Számos izolátumról megállapítottuk, hogy antagonistái e vizsgált baktériumfajoknak. Az antagonista fajok többsége széles spektrumú antibakteriális anyagot termelt, amely a kórokozók szaporodását gátolta.

Két izolátumot választottunk ki nagy és határozott szélű gátlási zónáik alapján a filloplánból a HIP32-es, a rizoszférából a HIR225-ös jelzésűt.

Meghatároztuk taxonómiai helyüket: a HIP32-es *Pantoea agglomerans*, aerogén G_1 biotípus, a HIR225-ös *Bacillus subtilis* faj volt.

A *Pantoea agglomerans* tenyészetszűrletének antibakteriális hatását is kimutattuk.

Az antagonista baktérium használhatóságának fontos szempontja annak fennmaradása természetes gazdanövényein. Szabadföldi körülmények között a tavaszi-nyári hónapok alatt a *Pantoea agglomerans* almafa lomblevelén fennmaradt és továbbszaporodott.

A *Xanthomonas vesicatoria*-val és a *Clavibacter michiganensis* subsp. *michiganensis*-szel fertőzött talajokban mindkét antagonista baktériumfaj redukálta a kórokozók sejtszámát.

A *Pantoea agglomerans* számos növényen, növényrészen (alma, körte, birs, Cotoneaster sp.) csökkentette a tűzelhalás tüneteinek súlyosságát. Hasonlóan gyérítő hatása volt a baktériumos foltok kialakulására paprikán és paradicsomon. Az antagonista hatás a kórokozót megelőző preventív kezelésben jelentős volt, mintegy 45%-ban csökkentette a betegség tüneteit. Paprikapalántákon nem csupán a xantomonászos betegség tüneteinek súlyosságát csökkentette, hanem megakadályozta a levelek hullását is. Az antagonistával kezelt növények nagyobb vigorral rendelkeztek.

Tanulmányomban kimutattuk, hogy a *Pantoea agglomerans* alkalmazása perspektivikus lehet az almatermésű növények tűzelhalásának, valamint a paprika és a paradicsom xantomonászos betegségének leküzdésére.

A *Bacillus subtilis* felhasználásával a xantomonászos és a klavibakteres talajfertőzöttség jelentősen csökkenthető.

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Appendix of biochemical characterizations

1. Potassium hydroxide solubility (KOH)

The KOH solubility test serves as a substitute for the Gram staining test. A few drops of 3% potassium hydroxide are put on a glass slide. The test colony of the epiphytic isolate (32) or soil isolate (225) from viable culture (24-48hrs) is picked from the plate with a sterile loop, stirred into the solution for 5-10 seconds on the glass slide. The loop is mixed and raised from the solution. If the solution is viscous enough to stick to the loop causing a thin strand of slime by lytic release of DNA, so the test is positive and the bacterium is Gram–negative. Absence of the slime consistence refers to a Gram positive bacterium (Suslow et al.,1982).

2. Cytochrome–oxidase (OX) test

The oxidase test was applied according to Kovács (1956). One platinum loop of a freshly grown bacterial culture of epiphytic and soil isolates 32 or 225 from Nutrient agar with 1% glucose were impregnated onto a filter paper moistened with a fresh 1% (w/v) aqueous solution of tetramethyl-p-phenylene-diamine dihydrochloride reagent (1g in 100ml isoamyl alcohol). A purple reaction in 10 sec. was considered as positive, and a purple reaction within 60 sec. or no reaction was considered as negative (Kovács,1956).

3. Glucose / oxidation / fermentation (O/F)

Hugh and Leifson medium was used for this test. A 10% glucose solution was filter sterilized and 0.5 ml aseptically added to the medium. Inoculates were prepared, two tubes for each of the antagonistic isolates. One was sealed with sterile melted paraffin to a depth of 2 cm over the growth while the others are not. A color change in the aerobic tube only (oxidation) indicates effective inoculation but no fermentation. A color change to yellow in the aerobic tube demonstrates glucose fermentation (Hugh and Leifson,1953, Sands,1990).

4. β-Galactosidase (ONPG)

β-Galactosidase releases ortho-nitrophenol from Ortho-nitrophenyl- β-D- Galactopyranoside (ONPG) This can be demonstrated by growing the epiphytic antagonist in ONPG broth. The ONPG solution contains 0.6% ONPG in 0.01M Na_2HPO_4 puffer. The release of yellow *O*-nitrophenol indicates β-Galactosidase activity. The color change may occur within 3hours of incubation at 37°C (Le Minor and Ben Hamida,1962).

5. Arginine dihydrolase (ADH)

A fresh culture of the tested antagonistic isolates 32 or 225 was stabbed into a soft agar tube of Thornley's 2A medium. Alkaline or positive reaction gave the medium a red color within 4 days due to the production of Ornithine, CO_2 and NH_3 from arginine (Thornley,1960).

6. Lysine decarboxylase (LDC)

According to the method of Moller (1955) 1% (w/v) of L (+)- lysine hydrochloride was incorporated in Moeller's Decarboxylase Broth Base medium, (see the appendix). The tubes were inoculated with the tested epiphytic antagonists followed by overlaying with sterile liquid paraffin, then incubated and examined daily for up to 4 days. As a result of bacterial fermentation of glucose in the medium, the indicator becomes yellow. The control tube, devoid of amino acids, will remain yellow; but a subsequent change to violet or purple in the tests indicates that alkaline degradation products have been produced in the course of decarboxylation of the respective amino-acid (Moeller,1955).

7. Ornithine decarboxylase (ODC)

According to the method of Moeller (1955) 1% (w/v) of L (+)-Ornithine hydrochloride was incorporated in

Moeller's Decarboxylase Broth Base medium (see the appendix). The tested tubes were inoculated with the tested epiphytic antagonist followed by overlaying with sterile liquid paraffin, then incubated and examined daily. Results of the color reaction are evaluated as in case of the LDC test (Moeller,1955)

8. Sodium citrate utilization (CIT)

Bacto Simmon's Citrate Agar is a medium capable of differentiation between members of *Enterobacteriaceae* and members of the genus *Bacillus.* Stabs were inoculated in Simmon's medium-slant tubes with antagonistic isolates HIP32 or HIR225 and gelled by addition of 2% agar, then incubated at 37^0C. Growth is indicated very clearly by colony formation and is usually accompanied by a color change of the indicator due to acid or alkali production indicating utilization of citrate (Simmons,1926).

9. Urease production (URE)

Urease catalyzes the breakdown of urea and release of ammonia into the medium. In an appropriately buffered broth medium the pH will rise to over 9.0 resulting in a color shift of the indicator dye used from yellow to magenta. Dye's medium (1968I) as in the appendix was inoculated with isolate 32 or 225. 0.016 g of cersol red, was added before medium sterilization and 200ml of 10% urea were added afterward. The same medium without urea was used as a check. Tubes were observed for 7 days (Sands,1990).

10. Tryptophane deaminase (TDA)

A TDA reagent was used for the detection of Tryptophane deaminase 3.4g of ferric chloride was dissolved in 100 ml of distilled water. By adding 1 drop of this reagent to the tested epiphytic antagonist color changes were recorded. Dark brown color indicates a positive reaction (Singer and Volcani,1955)

11. Indole production (IND)

Culture tubes containing; yeast extract; 5g/l and tryptone; 10g were inoculated with epiphytic isolate 32 or soil isolate 225 then incubated in a shaker at 27°C for 2-5 days. One ml of broth culture was added to 0.5 ml of Kovǎcs' reagent (75ml of warm amyl or isoamyl alcohol at 50^oC added to 5g of p-dimethyl-amino-benzaldehyde, when dissolved, when cool carefully add 25ml concentrated HCl) shaked well, and examined after about 1 min. The cherry red color reaction in the reagent layer indicated indole production (Sands,1990).

12. VOGES-PROSKAUER test (VP)

This test is modified from Benjaminson et al.,(1964) and detects the neutral products Acetoin (acetylmethylcarbinol) and 2,3-butanediol. Dye's medium (1968II) as in the appendix was inoculated with antagonistic isolates 32 or 225. Cultures were shaked, then incubated at 27°C and tested after 2 - 5 days. One ml samples were combined with 0.6 ml of alpha-naphthol (5% w/v in 100% ethanol) in a tube, 0.2 ml of 40% KOH was added and shaken vigorously for up to two hours. Positive reaction was evidenced by a pink color (Sands,1990).

13. Gelatin hydrolysis (GEL)

Nutrient agar with 0.4% (w/v) gelatin was poured into Petri dishes, cooled and dried. Plates were inoculated with isolates 32 or 225 then incubated at 27°C for 3 days. The plate surface was then flooded with 5ml of mercuric chloride (Toxic) solution (12g/ HgC; 80ml of distilled water and 16ml of concentrated HCl). A clear zone surrounding bacterial growth indicates positive reaction for gelatin hydrolysis (Kohn,1953, Dickey and Kelman,1988) .

14. Carbohydrate utilization

Acid production from carbohydrates was tested in agar tubes with medium C of Dye's (1968) as in the appendix. A filter sterilized concentrated solution of a carbon source ((glucose (GLU), mannitol (MAN), Inositol (INO), Sorbitol (SOR), Rhamnose (RHA), sucrose (SAC), melibiose (MEL), Amygdalin (AMY), arabinose (ARA)) was added

aseptically to a final concentration of 0.5% (w/v). Medium was inoculated with the epiphytic antagonist. As glucose utilization is a common positive check, tubes without carbon source were added as a negative check (purified agar). All tubes were incubated at 27°C and examined for acid production (yellow color) after 2, 4 ,7 ,21 and 28 days (Hugh and Leifson,1953).

15. Nitrate reduction (NO_2-NO_3)

The medium of Fahy and Hayward was used for this test. Tubes were stab inoculated with a loop of the test antagonist 32 or 225. Vigorous-growth in 5 days at 27°C is an indicator of denitrification. Another test can be used and applied as described in the Instruction manual of API20 E. tests, version A. Add 1 drop of each of **NIT1** (sulfanilic acid, 0.8g; acetic acid 5N/100ml) and **NIT2** (N-N-dimethyl-1-naphthylamine, 0.6g;acetic acid 5N/100ml) reagents to the GLU tube. Wait 2-3 minutes. A red color indicates a positive reaction. A negative reaction (yellow) may be due to the reduction to nitrogen (as sometimes evidenced by gas bubbles): add 2-3 mg of Zn (Zinc dust, 10g) reagent to the GLU tube. After 5 minutes, if the tube remains yellow it indicates an N_2 positive reaction, if the tube turns pink-red, an N_2 negative reaction occurred (Sands,1990).

16. Lactose fermentation

MacConkey agar medium was used for this test. Dried plates were serial-inoculated with the epiphytic antagonist and incubated for 16-18h at 36°C. The lactose fermentation test gives a more clear-cut differential between these enteric organisms and the lactose fermenting Gram-negative bacilli, by promotes their development. The medium may be brick in color and sometimes surrounded by a zone of precipitated bile due to the action of the acids produced by fermentation of lactose, upon the bile salts and the subsequent absorption of neutral red (Difco Manual,1953, Barrow, and Feltham,1993).

17. Catalase test

To 1ml of a 3 % solution of hydrogen peroxide on a slide add a loop of fresh culture of the tested soil isolate. Bubbles indicate a positive catalase test (Sands,1990).

18. Starch hydrolysis

Nutrient agar plates containing 0.2% (w/v) soluble starch were streaked with a culture of tested soil isolate and incubated at 28°C until heavy growth occurred. Plates were then flooded with KI solution (Iodine, 1.0g; Potassium iodide, 2.0g/100ml of distilled water, 100ml). A clear zone around a colony is a positive reaction for starch hydrolysis while starch stains blue or black (Sands,1990)

9. Casein hydrolysis

Casein agar (Milk Agar) medium was used for this test. 500ml; skim milk, and 500ml of double-strength nutrient agar were sterilized by heating at 115^{0}C for 10min. After cooling to about 50°C, double-strength Nutrient agar melted and cooled to 50-55°C then added to the skimmed milk. The medium was rapidly mixed and distributed in Petri dishes or tubes. Soil isolates were stab inoculated and incubated for 7-days. Proteolysis was observed as a clear zone surrounding the colonies indicating a positive reaction of the test (Barrow,. and Feltham,1993):)

20. Malonate utilization

The malonate-phenylalanine medium of Shaw and Clarke (1955) was prepared by dissolving the solid ingredients in water by heating, followed by filtering and addition of the indicator solution, and sterilization at 115^{0}C for 20min. The medium was lightly inoculated with the soil isolate tested and incubated for 18-24h. Cultures kept for the phenylalanine deamination test. Color change was examined. A positive malonate reaction was indicated by a deep blue color, a negative reaction by the unchanged greenish color of the medium (Barrow and Feltham,1993)

21. Hydrogen sulphide production

A tube of Triple sugar Iron Agar medium (TSI) was inoculated by stabbing the butt and streaking the slope with the tested soil isolate and observed daily for up to 7days. Blackening of the butt due to H_2S production. Some organisms produce only a dark pigment on the slope and this should not be mistaken for a positive result (Barrow and Feltham, 1993)

22. Aesculin hydrolysis test

Aesculin broth was used for this test. Aesculin, 1g; ferric citrate 0.5g; and Peptone water, 1000ml. After dissolving the Aesculin and iron salt in the Peptone water, the solution was sterilized at 115^oC for 10 min. The resulting medium was inoculated with the tested soil isolate and gelled by the addition of 2% agar and incubated at 27^oC for 7days(Barrow and Feltham, 1993).

Appendix of table of API 20 E microtests and substrates

Codes of tests	Detected enzymes/ reaction	Substrates
ONPG	Beta-Galactosidase	Ortho-nitro-phenyl-galactoside
ADH	Arginine dihydrolase	Arginine
LDC	Lysine decarboxylase	Lysine
ODC	Ornithine decarboxylase	Ornithine
CIT	Citrate utilization	Sodium citrate
H2S	H_2S Production for cysteine	Sodium thiosulphate
URE	Urease production	Urea
TDA	Tryptophane deaminase	Tryptophane
IND	Indole Production	Tryptophane
VP	Acetoin	Sodium pyruvate
GEL	Gelatine hydrolysis	Kohn's gelatin
GLU	Carbohydrate utilization (Oxidation / fermentation(OF))	Glucose
MAN		Mannitol
INO		Inositol
SOR		Sorbitol
RHA		Rhamnose
SAC		Sucrose
MEL		Melibiose
AMY		Amygdalin
ARA		Arabinose
OX	Cytochrome oxidase	Tetramethyl-p-phenylene-diamine dihydrochl
NO3-No2	NO_2 production	N-N-dimethyl-1-naphthylamine
MOB	Motility	Scanning E. microscopy
MAC	Lactose fermentation	Lactose

Appendix of biological control products

The following are the commercially available products for biological control of plant pathogenic bacteria

including only microbial products (containing living organisms) labeled for disease control

1. Galltrol-A

Biocontrol Agents*:* *Agrobacterium radiobacter* strain 84

Target pathogen : *Agrobacterium tumefaciens* causing crown galls

Crop*:* fruit, nut, and ornamental nursery stock

Formulation: petri plates with pure culture grown on agar

Manufacturer: Ag BioChem, Inc. CA-USA.

2. Nogall, Diegall

Biocontrol Agent: *Agrobacterium radiobacter*

Target crop/ pathogen: *Agrobacterium tumifaciens*

Crop: trees, : fruit, nut, and ornamental nursery stock

Formulation: washed plates; culture suspensions

Manufacturer: Bio-care Technology , NSW, Australia

3. Norbac 84C

Biocontrol Agent: *Agrobacterium radiobacter* strain k84

Target pathogen/Disease: *Agrobacterium tumefaciens*

Crop: fruit, nut, and ornamental nursery stock

Formulation: aqueous suspension containing bacterial cells, methylcellulose, and phosphate buffer (refrigerate)

Manufacturer: new BioProducts, OR-USA.

4. Conquer

Biocontrol Agent*:* *Pseudomonas fluorescens*

Target pathogen/Disease: *Pseudomonas tolassii*

Crop: mushrooms

Formulation: aqueous suspension containing bacterial cells,

Manufacturer: Sylvan Spawn Laboratory, West hills Industrial Park, Kittanning, PA-USA.

5. System 3

Biocontrol Agent: Bacillus subtilis GB03 and chemical pesticides

TargetCrop/ pathogen: seedling pathogens

Crop: barley, beans, cotton, peanut, pea, rice, soybean
Formulation: dust
Manufacturer: Helena Chemical Co., Memphis, Tn, USA.

6. Victus

Biocontrol Agent: *Pseudomonas fluorescens* strain NCIB 12089
Target pathogen/ Disease: *Pseudomonas tolassii* that causes bacterial blotch disease
Crop: mushrooms
Formulation: aqueous suspension of fermentor biomass
Manufacturer: Sylvan Spawn Laboratory, West Hills Industrial Park, Kittanning, PA- USA

7. PSSOL

Biocontrol Agent: *Pseudomonas solanacearum* (nonpathogenic)
Target pathogen/Disease: *Pseudomonas solanacearum*
Crop: vegetables
Manufacturer: Natural Plant Protection, Nogueres,France.

8. Serenade

Biocontrol Agent: *Bacillus subtilis*
Target Crop/ pathogen: powdery mildew, downy mildew, Cercospora leaf spot, early blight, late blight, brown rot, fire blight, and others
Crop: cucurbits, grapes, hops, vegetables, peanuts, pome and stone fruits. **Formulation:** wettable powder
Manufacturer: Davis, CA USA Agra Quest, Inc.

9. Blightban A506

Biocontrol Agent: *Pseudomonas fluorescens* A506
Target pathogen/Disease: Frost damage, *Erwinia amylovora* disease,
Crop: almond, apple, apricot, blueberry, cherry, peach, pear, potato, strawberry, tomato
Manufacturer: Plant Health Technologies, Santa Ana, Fresno, CA-USA

10. Phagus

Biocontrol Agent: bacteriophage
Target pathogen/Disease:*Pseudomonas tolassii*

Crop: *Agaricus* spp., *Pleurotus* spp.

Formulation: bacterial suspension

Manufacturer: Natural Plant protection, Nogueres, france .

Others are not in commercial products list and also they have promising effects as biocontrol agents:.

11. ***Erwinia herbicola (Pantoea agglomerans)*** **strains:**

Biocontrol Agent: *Erwinia herbicola* or *Pantoea agglomerans*

Target Crop/ pathogen: fire blight, pathogen, *E. amylovora* and others

Crop: **P**ome fruit trees, stone fruit trees, and others, but it is still under experimental tests and in small areas applications as commercial bioproduct.

12. ***Bdellovibrio bacteriovorus*** against *Pseudomonas syringae pv. glycinea*. The causal of bacterial blight of of Soybean.

13. ***Pseudomonas fluorescens*** sold as Dagger G for use against *Rhizoctonia* and *Pythium* damping off of cotton.

Appendix of the media

Basal medium (Ayer's et al., 1919):

(NH_4)-H_2PO_4; 500mg, KCl; 0.1g, $MgSO_4.7H_2O$; 0.1g, Distilled water,500ml.

Dye's medium (1968I):

NH_4HPO_4, 0.5g; K_2HPO_4, 0.5g; $MgSO_4.7H_2O$, 0.2g;NaCl, 5g; yeast extract (Difco), 1.0g; cersol red, 0.016 g and water, 800ml at (pH 6.8).

Dye's medium (1968II):(g/l)

Glucose, 5.0g; (NH_4 H_2PO_4, 0.5; K_2HPO_4, 0.5 g; $MgSO_4$ $7H_2O$, 0.2 g; NaCl, 5g; yeast extract (Difco), 5g.

Dye's medium C(1968):

(NH_4 H_2PO_4, 0.5; K_2HPO_4, 0.5 g; $MgSO_4$ $7H_2O$, 0.2 g; NaCl, 5g; yeast extract (Difco), 1g; agar(Difco or Noble) 12g;water,1l; brom-cresol purple, 0.7 ml of 1.5% ethanol solution).

Fahy and Hayward,1983:

In (g/l): peptone (difco) 10g; NaCl 5.0g; KNO_3 2.0g; and agar(difco),3.0g; (pH to 7.0).

King's medium B(KB):

Protease peptone,20g; glycerol,10g; K_2HPO_4,1.5g; $MgSO_4.7H_2O$,1.5g; agar,15g; distilled water,1 liter (PH 7.2)

Koser' medium:

NaCl, 5g; $MgSO_4.H_2O$, 0.2g; $NH_4H_2PO_4$,1g; K_2HPO_4,1g; Citric acid, 2g; Distilled
water,1000ml.

Moeller's Decarboxylase Broth Base medium:

Containing per liter of distilled water: peptone,5g; yeast extract,3g; glucose,1g; 0.2% solution of bromocresol purple,10ml,

Malonate-phenylalanine medium:

$(NH_4)SO_4$, 2g; K_2HPO_4, 0.6g; KH_2PO_4, 0.4; NaCl,2g; Sodium malonate, 3g; DL-phenylalanine, 2g; Yeast extract,2g; Distilled water1000ml; Bromthymol blue,

MacConkey agar medium: In (1/liter)

Bacto-peptone; 17gr, Proteose Peptone, Difco;3g, Bacto-lactose; 10g, or Bacto-Bile salts No. 3; 1.5g, sodium chloride; 5.0g, Bacto-neutral red; 0.03g, Bacto-agar; 13.5g,Bacto-Crystal Violet; 0.001g.

Medium of Hugh and Leifson,1953:

In (g/l): Base peptone, 2g.; NaCl, 5.0g; H_2PO_4, 0.3g;agar (Difco), 3.0g; and 3 ml/l of 1% aqueous solution of Bromthymol blue(0.03g or15ml of 0.2%of the solution).

Nutrient Agar (Merck): (NA) typical composition:

20g/1liter distilled water; meat extract; 3.0g, yeast extract (oxoid) 0.2%; peptone from meat; 5.0g, agar-agar; 12.0g(pH 7.0-).

Nutrient broth:

g/L; peptone from meat; 5.0g, meat extract;3.0g

Potato Dextrose Agar (PDA):g/L

glucose,20g; potato extract,4g; bacteriological agar,15g; distilled water;1liter.

Potato dextrose broth (PDB) medium:

(4g / l Potato extract, 20g / l dextrose).

Simmon's Citrate Agar:

composed of: Magnesium sulfate; 0.2g, Monoammonium phosphate; 1g, Dipotassium phosphate; 1g, Sodium Citrate;2g, Sodium Chloride; 5g, -Agar; 15g, -Brom thymol Blue; 0.008%, at 0.08g; 1liter distilled water.

Thornley's medium 2A:

peptone, 1g; NaCl,5g; K_2HPO_4, 0.3g; agar,3g; phenol red,0.01g; L(+)-arginine HCl,10g, 1liter distilled water.

Triple Sugar Iron Agar (TSI) medium:

Meat extract, 3g; Yeast extract, 3g; Peptone, 20g; Glucose1g; Lactose, 10g; Sucrose,10g; $FeSO_4.7H_2O$, 0.2g; NaCl,5g; $NaS_2O_3.5H_2O$, 0.3g; Agar,20g;

Yeast Dextrose Chalk Agar (YDC):

Yeast extract,10g; D-glucose 20g; precipitated chalk($CaCO_3$), 20g; oxoid agar No.3,12g; distilled water, 1liter;(pH7.2).

Appendix of statistical analysis

```
          MiniStat 3.2 -- Copyright: Vargha Andr s, 1999
 Jogosult felhasznál:
      Ferenczy Antal Zoltán, Szent István Egyetem KTK MIT Budapest

Input f jl: LEAF.MST, Output f jl: LEAF.OUT

           Statisztikai rutin: Alapstatisztikák
     -----------------------------------

           Elemzés sorszáma = 1

  Feltételes csoportok defin ci ja

    csop./Treat   Kód       Név
         --------------------------------
          1.        1    'Treat=1'
          2.        2    'Treat=2'
          3.        3    'Treat=3'

    Csoportindex: 1.  Csoportnév: 'Treat=1'

     Beolvasott esetek száma . . . . . . .  48
    Aktuális csoport elemszáma  . . . . .  16

                                Változ˘  Érvényes          Vari ci˘s   Legkisebb    Legnagyobb
  Ind N,v     esetek   Átlag   szórs  együtth.  ,rt,k z-,rt,k  Ert,kézás-,rt,k
----------------------------------------------------------------------
                                                                     -
                                 2 Area      16   5.223   3.024   0.579   1.630  -1.19   12.31   2.34
                                  3 Number     16   3.313   1.138   0.344      1  -2.03       5   1.48
----------------------------------------------------------------------
                                                                     -
    Csoportindex: 2.  Csoportnév: 'Treat=2'

     Beolvasott esetek száma . . . . . . .  48
    Aktuális csoport elemszáma  . . . . .  16

                                Változ˘  Érvényes          Vari ci˘s   Legkisebb    Legnagyobb
     Ind Név     esetek   Átlag   szór s  együtth.  ,rt,k z-,rt,k  ,rt,k z-,rt,k
----------------------------------------------------------------------
```

-
2 Area 16 8.361 3.255 0.389 3.630 -1.45 14.06 1.75
3 Number 16 6.500 1.317 0.203 4 -1.90 8 1.14

-

Csoportindex: 3. Csoportnév: 'Treat=3'

Beolvasott esetek száma 48
Aktuális csoport elemszáma 16

Változ "Érvényes Vari ci˘s Legkisebb Legnagyobb
Ind Név esetek Átlag szórs együtth. ‚rt‚k z-‚rt‚k ‚rt‚k z-‚rt‚k
----
-
2 Area 16 8.287 4.711 0.568 3.350 -1.05 21.11 2.72
3 Number 16 6.250 1.770 0.283 3 -1.84 9 1.55

Input f jl: LEAF2.MST, Output f jl: LEAF2.OUT

Statisztikai rutin: Független mint k egyszempontos ”sszehasonl˘t sa
----
Elemzés sorszáma = 1

Beolvasott esetek száma. 48
Érvényes esetek száma. 48

Jel”l‚s: +: p < 0.10 *: p < 0.05 **: p < 0.01

Függ“ változ˘: Area (2)

Csoportos˘t˘ változ˘: Treat

Csoport Index	Név	Érvényes esetek	Átlag	szór s	Minimum	Maximum
1.	Treat=1	16	5.223	3.024	1.630	12.31
2.	Treat=2	16	8.361	3.255	3.630	14.06
3.	Treat=3	16	8.287	4.711	3.350	21.11

Elm‚leti szór sok egyenl“s‚g‚nek tesztel‚se
- O'Brien-próba: F(2,45) = 1.088
- Levene-próba: F(2,45) = 1.709

Elm‚leti tlagok egyenl“s‚g‚nek tesztel‚se
Hagyom nyos elj r s, amely felt‚telezi a sz˘r shomogenit st:
- Varianciaanal˘zis: F(2,45) = 3.670*
Hatasvariancia = 51.3065, Hibavariancia = 13.9786
Korrel ci˘s h nyados (nemline ris korrel. együtthat˘): e = 0.374

Robusztus elj r sok, amelyekn‚l nem sz ks‚ges a sz˘r shomogenit s:
- Welch-pr˘ba: W(2,29) = 4.649*
- James-próba: U = 9.511*
- Brown-Forsythe-pr˘ba: BF(2,38) = 3.670*

Az Átlagok Tukey-Kramer-f,le p ronk,nti ”sszehasonl˘t sa (k = 3, f = 45):

T12= 3.36+ T13= 3.28+ T23= 0.08

Függ“ változ˘: Number (3)

Csoportos˘t˘ változ˘: Treat

Analysis of Variance of tomato experiment

Pre-treatment

ÖSSZESÍTŐ TÁBLA

Regressziós statisztika	
r értéke	0,695593
r-négyzet	0,48385
Korrigált r-négyzet	0,459272
Standard hiba	0,478724
Megfigyelések	23

VARIANCIAANALÍZIS

	df	*SS*	*MS*	*F*	*F szignifikanciája*	
Regresszió	1	4,511545	4,511545	19,68586	0,000229	
Maradék	21	4,812716	0,229177			
Összesen	22	9,324261		at F5%; 1,22= 4,3<19,69		Significant effect

	Koefficiensek	*Standard hiba*	*t érték*	*p-érték*	*Alsó 95%*	*Felső 95%*	*Alsó 95,0%*	*Felső 95,0%*
Tengelymetszet	1,91204	0,184161	10,38245	1E-09	1,529057	2,295024	1,529057	2,295024
X változó 1	0,168011	0,037867	4,436875	0,000229	0,089263	0,24676	0,089263	0,24676

c	6,766881
b	0,168011
A	4

Post-treatment

ÖSSZESÍTŐ TÁBLA

Regressziós statisztika	
r értéke	0,810035
r-négyzet	0,656157

Korrigált r-négyzet	0,639783
Standard hiba	0,510445
Megfigyelések	23

VARIANCIAANALÍZIS

	df	SS	MS	F	F szignifikanciája
Regresszió	1	10,44153	10,44153	40,07435	2,82E-06
Maradék	21	5,471634	0,260554		
Összesen	22	15,91317		at F5%;	40,07> 4,3 (as control no sign. Effect)

	Koefficiensek	Standard hiba	t érték	p-érték	Alsó 95%	Felső 95%	Alsó 95,0%	Felső 95,0%
Tengelymetszet	2,339728	0,196363	11,91529	8,3E-11	1,931368	2,748088	1,931368	2,748088
X változó 1	0,255598	0,040376	6,33043	2,82E-06	0,171632	0,339565	0,171632	0,339565

c	10,37841
b	0,255598
A	5

Control

ÖSSZESÍTŐ TÁBLA

Regressziós statisztika	
r értéke	0,810035
r-négyzet	0,656157
Korrigált r-négyzet	0,639783
Standard hiba	0,510445
Megfigyelések	23

VARIANCIAANALÍZIS

	df	SS	MS	F	F szignifikanciája	
Regresszió	1	10,44153	10,44153	40,07435	2,82E-06	
Maradék	21	5,471634	0,260554			
Összesen	22	15,91317			at F 5%;	40,07> 4,3 (no sign. Effect)

	Koefficiensek	Standard hiba	t érték	p-érték	Alsó 95%	Felső 95%	Alsó 95,0%	Felső 95,0%
Tengelymetszet	2,339728	0,196363	11,91529	8,3E-11	1,931368	2,748088	1,931368	2,748088
X változó 1	0,255598	0,040376	6,33043	2,82E-06	0,171632	0,339565	0,171632	0,339565

c	10,37841
b	0,255598
A	5

Appendix of tables

Table 1. Effect of *Pantoea agglomerans* on pre- and post-treated tomato seedlings inoculated with *Xanthomonas vesicatoria* strain SO8 during December and January.

December. – January.1998-1999					
Days after symptom appearance	**Pre-treatment**	**Control**	**Post-treatment**	**Disease reduction % in Pre-treatment**	**Disease Reduction % in Post-treatment**
1	1.00	1.50	1.20	40	20
2	2.00	3.10	2.30	35	26
3	2.00	3.10	2.36	38	24
4	2.20	3.38	3.00	34	11
5	**2.50**	**3.62**	**3.15**	**34**	**14**
6	2.70	3.83	3.83	30	9.0
7	3.00	4.00	3.65	25	9 .0

Table 2 . Effect of *Pantoea agglomerans* on pre- and post-treated tomato seedlings inoculated with *Xanthomonas vesicatoria* strain SO8 during February and March

February. - March. 2000					
Days after symptom appearance	**Pre-treatment**		**Post-treatment**	**Disease reduction % (Pre-treatment)**	**Disease reduction % (Post-treatment)**
1	1.99	2.33	2.08	17	12
2	2.30	2.90	2.50	26	16
3	2.50	3.33	3.00	33	11
4	2.80	3.83	3.50	36	11
5	3.00	4.00	3.50	35	11
6	3.10	4.20	3.83	35	10
7	3.50	4.70	4.50	34	12

Table3. Effect of *P. agglomerans* on pre and post-treated tomato seedlings inoculated with *Xanthomonas vesicatoria* during May and June

May – June1999					
Days after symptom appearance	**Pre-treatment**	**Control**	**Post-treatment**	**Disease Reduction % in Pre-treatment**	**Disease Reduction % in Post-treatment**
1	1.50	2.30	2.20	50	12
2	1.70	2.33	2.25	10	6.0
3	2.25	3.20	3.00	30	6.0
4	2.30	3.40	3.33	12	2.0
5	2.50	3.83	3.50	**12**	**9.0**
6	2.80	3.83	3.50	12	9.0
7	3.00	4.80	4.00	36	10

<u>Table .</u> Percentage of disease reduction in tomato plants treated by *Pantoea agglomerans* at different leaf developmental stages

Advanced stage of seedlings	Days after symptoms appearance	Disease reduction %	
		Pre-treatment	Post-treatment
A	1	45	24
B		30	12
C		14	10
D		20	19
E		21	17
A	2	43	22
B		25	8.0
C		28	0.0
D		20	13
A	3	39	20
B		20	8.0
C		31	11
A	4	38	16
B		22	10
C		26	5.0
A	5	38	14
B		20	9.0
C		25	10
A	6	35	12
B		20	9.0
A	7	36	13
B		22	9.0
A	8	36	12
B	8	22	10
Control		100	100

Advanced stages of tomato leaves: A= 6 leaves, B=10 leaves, C, D, and E = 12leaves