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

THESIS OF Ph.D. DISSERTATION

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1. ANTECEDENTS AND OBJECTIVES OF WORK

Biological control of plant pathogenic bacteria is the latest method and alternative tool to application of chemicals in order to exclude or reduce the effect of pathogens. During the last four decades efforts have been continued to improve biological control agents as commercial products for use in disease control (Baker and Cook (1974), Cook and Baker (1983), Cook (1993).

The effectivity of biological control agents on numerous plant-pathogen combinations was demonstrated by several authors (Tzeng et al.(1994), Nishioka et al. (1997), Biró et al.(1998), and Vanneste (2000),

Production of antimicrobial metabolites by selected antagonists was also confirmed by Amellal et al. (1998) and Vanneste, (2000). Kearn and Hale (1995) demonstrated persistance and establishment of antagonists in its orginal host apple plant. Reduction of fire blight disease severity by antagonistic bacteria was demonstrated by Vanneste,(1996, 2000).

Bacillus subtilis is well known as biocontrol agent in soil conditions (Bochow, 1992). *Pantoea agglomerans* is an epiphytic as well as a soil inhabitants too Brenner (1983) and Vanneste (2000)

The strategy of this study has been to find antagonists against different types of diseases in economically important crops such as fire blight caused by *Erwinia amylovora* in pomaceous species Hevesi (1996) and Németh, (1999), bacterial spots diseases caused by *Xanthomonas vesicatoria* strains in pepper and tomato and bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis* in tomato in order to reduce the losses caused by these diseases Klement 1959 (in. Ubrizsy,1965).

Different control measures were used in Hungary too against these diseases but still are insufficient enough to reduce the losses they cause.

Biological control programs are developed all over the world to produce biological products that could effectivly reduce losses using

different types of microorganisms Vajna (1987), Aponyi-Garamvölgyi (1989), Dormanns-Simon et al. (1997), Vanneste (2000).

Although biological control of bacterial plant diseases with antagonistic bacteria is still insufficiently practiced in fields all over the world as do other control measures, but it has been considered promising in its initial research programs because of its safety to the environment.

The present study was conducted to select identify and test the effectivity of biological control agents against the above diseases as a first study in Hungary.

Within these investigation frameworks, the following objectives had been carried out:

- Isolation of antagonistic bacteria from the phylloplane and from the rhizosphere (using different soil samples from Hungary and Libya)

- Application of *in vitro* methods for evaluation of effectivity of these antagonistic isolates to select 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 Laboratory and greenhouse conditions

- Persistence and establishment of the antagonists on foliage of apple trees for possible utilization of biocontrol agents in field conditions.

2. MATERIALS AND METHODS

For **isolation of antagonistic bacteria from the phylloplane** of leaves and unripe fruits of apple trees cultivar 'Starking' and from the rhizosphere of soil samples collected from different locations in Hungary and Libya. The following plant pathogenic bacteria were tested: *Clavibacter michiganensis* subsp. *michiganensis,* the causal agent of bacterial canker of tomato, *Xanthomonas vesicatoria,* the causal agent of bacterial spot of pepper and tomato, and *Erwinia amylovora,* the causal agent of fire blight of pomaceous plants as selected diseases for this study. In addition, the following plant pathogens were also served for selection, effectivity and **confirmation of antibacterial spectra** of the biological control agents:, *Agrobacterium vitis* (the causal agent of crown gall and cankers of grapes, *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 hortorum* pv. *pelargonii* (the causal agent of spotting and wilting of pelargonium), *Xanthomonas axonopodis* pv. *phaseoli (*the causal agent of common blight of beans) and different pathovars of *Pseudomonas syringae* (the causal agent of spots, blight and canker diseases in different hosts)

Antifungal activity was also detected towards some isolates of plant pathogenic fungi such as *Geotrichum candidum* and some formae speciales of *Fusarium oxysporum.* Different types of media such as Nutrient agar, Potato dextrose agar, King B, YDC and selective media supplemented with antibiotics were used during this study.

For isolation and selection of antagonists from both phylloplane and rhizosphere, **double layer soft agar plate technique and streaking methods** were used for testing the antagonistic effect of selected isolates and evaluation of inhibition zones caused by the antagonists on field of pathogens were measured in mm. Two strains coded as Hungarian phylloplane and rhizosphere isolates (HIP32 and HR225) were selected

according to their strong inhibition effects to most plant pathogenic strains tested and the wide spectra of these effects.

Time-dependence of metabolite production of HIP32 from 1 - 7 days on the above pathogens was clarified.

For **investigation the metabolite production** in culture filtrate, the epiphytic antagonist HIP32 was cultivated in Potato Dextrose Broth (PDB) and Ayer's basal medium for 7days. The effectivity of the extracted culture filtrate in agar diffusion tests at concentrations: 1:12.5, 1:25, and 1:50 to *Erwinia amylovora, Clavibacter michiganensis* subsp. *michiganensis,* and *Xanthomonas vesicatoria* was proved onto PDA plates using the double agar plate technique.

Minimal inhibitory effects of the culture filtrate in poisoned agar plate were also determined by mixing the above dilutions to PDA medium and then applying the indicator pathogens. The survival colony forming units of these pathogenic strains were counted.

The persistence and establishment of epiphytic antagonistic isolate HIP32 were monitored in apple trees cultivar 'Starking' in field conditions by spraying the foliage with the suspension of the epiphytic isolate in three independent experiments at different seasonal times. Leaf samples were taken periodically for 4 weeks and numbers of colony forming units of the antagonist (CFU / ml^{-1}/cm^2 of apple leaf area) were determined after re-isolated on selective medium supplemented with antibiotic Trimethoprim.

Confirmations of non-pathogenic characters of the selected antagonists were carried out by inoculations in tobacco, pepper, tomato plants and apple leaves. Harmlessness was evaluated by absence of disease symptoms and hypersensitive reaction in tobacco leaves using different concentrations of the antagonistic suspension. Soft rot tests in potato slices were conducted to evaluate the non-pectolytic activity of the antagonists.

Cultural and morphological characterization of HIP32 and HIR225 isolates were carried out by determine the presence and type of flagella

using an electron microscope. Gram character by KOH test; spore forming ability by heating of liquid culture. For colony type, and growth of the isolates on PDA, NA, King B, and YDC media were used.

Biochemical characterization involved the employment of the cytochrome oxidase, oxidation/fermentation (O/F) of glucose for both isolates (Sands In: Klement et al.,1990) were made. API 20 E containing 20 different biochemical microtests (for *Enterobacteriaceae*) were used for the epiphytic isolate HIP32 (Ewing and Fife, 1972, Goor et al.,1984 and Gavini et al.,1989),

Tests such as Catalase, Starch, Aesculin, and Casein hydrolysis, H_2S production (on TSI medium), and Malonate utilization (on malonate phenylalanine medium) were carried out for the soil isolate (Barrow and Feltham ,1993).

Quantitative analysis of survival of pathogens in the soil under the influence of the antagonists. For re-isolation of the pathogens from soils treated by the antagonists selective media were prepared. Testing the pathogenic strains of *Xanthomonas vesicatoria*, *Clavibacter michiganensis* subsp. *michiganensis*, the antagonists and mixture of undetermined soil microflora in the sensitivity tests to different antibiotics demonstrate that Nitrofurantoin is useful for re-isolation of *Xanthomonas vesicatoria* strains Xv14, SO8 and Lincomycin for re-isolation of *Clavibacter michiganensis* subsp. *michiganensis.* The antagonistic isolates HIP32 and HIR225 and tested soil microflora were sensitive to these antibiotics.

The effect of epiphytic and soil antagonistic isolates was evaluated against *Xanthomonas vesicatoria* strain SO8 and *Clavibacter michiganensis* subsp. *michiganensis* strain Cm3 using soil sample type Őrbottyán. Numbers of colony forming units/g of soil of these pathogens in the presence of antagonists were monitored by weekly samplings on the above-described selective media.

Fire blight disease reduction by the epiphytic antagonist HIP32. Tests were carried out in leaf discs of apple cultivars: 'GRANNY SMITH', 'STARKING', 'LIBERTY', 'SPARTAN', 'PRIMA', 'CSÁNYI-M9', 'IDARED / M4', 'MUTSU / M9', 'IDARED / M9' and 'FREEDOM / MM 106' by pre- and post-treatments. The discs were dipped into suspension of the epiphytic antagonist then inoculated with *Erwinia amylovora* (pre-treatment), and alternatively, applying the epiphytic antagonistic strain 24 hr after the inoculation by the pathogen (post-treatment). Complete leaves, flowers, and fruits of selected pomaceous plants of *Malus domestica*, *Pyrus communis, Cydonia oblonga,* and also flowers of *Cotoneaster sp.* were dipped into suspension of the epiphytic antagonist ($5x10^8$ cfu ml^{-1}) and of *Erwinia amylovora* Ea23, Ea17, Ea1, and Ea29 strains using the above mentioned method.

Reduction of leaf spot disease of tomato and pepper by epiphytic antagonist HIP32 was conducted in greenhouse experiments using seedlings of tomato-cultivar 'Kecskeméti 262' and pepper cultivar 'Cecei SH' inoculated with the leaf spot causal agent *Xanthomonas vesicatoria* strains SO8 in tomato, and Xv14 in pepper

HIP32 isolate was applied 24 hr before the pathogen inoculation as pre-treatment and afterwards in reverse as post-treatment. These different experiments were independent from each other and represented different developmental stages of test plants. Visual disease symptoms were reported when symptoms appeared using categories of disease rating and statistically analysis.

Besides measuring the rate of disease reduction in pepper seedlings the development of leaves (the leaf size and numbers) was also determined.

The epiphytic HIP32 isolate was applied 24hr before and after inoculation with *Xanthomonas vesicatoria* strain Xv14 and measurements were made 2 weeks after.

The length of persistence of protective effect of the epiphytic antagonistic isolate HIP32 was also evaluated on pre-treated pepper plants until 7th day, followed by inoculation with *Xanthomonas vesicatoria* strain Xv14 in each successive day.

3. RESULTS

- **Isolation and selection of antagonists** from the phylloplane of apple trees and from the soil rhizosphere confirmed the presence of numerous antagonistic bacterial species effective against most strains of phytopathogenic bacterial and fungal species tested. Results also showed that many epiphytic and soil isolates were antagonistic against different strains of *Erwinia amylovora* the causal agent of fire blight of pomaceous species, strains of *Xanthomonas vesicatoria* the causal agent of bacterial spot of tomato and pepper, and *Clavibacter michiganensis* subsp. *michiganensis* the causal agent of bacterial canker of tomato.
- **Antimicrobial spectra of the antagonistic isolates** showed wide spectra and clear effect of the selected antagonist HIP32 to the tested pathogenic strains. Antifungal activity was also demonstrated against *Geotrichum candidum* and some formae speciales of *Fusarium oxysporum*. Differences in sensitivity of the tested pathogenic species and strains were observed to the effect of the antagonistic isolates but no resistant strains were found.

From the investigated epiphytic isolates of apple foliage that had antagonistic effects on the basis of *in vitro* tests, one epiphytic isolate coded as (HIP32) was selected according to its strong and wide antibacterial spectrum for further characterization and testing. Among the different antagonistic soil isolates that had strong and wide antimicrobial effects strain HIR225 was selected for further experimental work.

- **Evaluation of effectivity** and the activity of the antagonistic isolate HIP32 as biocontrol agent was confirmed by metabolite production in

culture filtrate (CF) against the indicator pathogenic strains (*Erwinia amylovora, Clavibacter michiganensis* subsp. *michiganensis,* and *Xanthomonas vesicatoria)*. Colony forming units were also counted in poisoned agar plate and confirmed the effect of CF against these indicator pathogens. Basal medium was found to be more effective than PDB in stimulating the activity of antagonistic isolate HIP32 and the production of its metabolites in culture filtrate.

- **Time-dependent effect** on metabolite production of epiphytic isolate HIP32 was determined. The activity was higher after the 6^{th} and 8^{th} days of cultivation as compared to shorter incubation.

The persistence and establishments of isolate HIP32 on apple leaves was successfully achieved periodically from May to August and also the multiplication was observed by re-isolation on selective Trimethoprim-containing medium. High activity and more abundant presence of the epiphytic isolate in summer rather than in spring periods were clarified.

- **Non-pathogenic ability** of the epiphytic HIP32 and soil HIR225 isolates was proved in tobacco, tomato, pepper, and apple leaves and by hypersensitive reaction (HR) in tobacco leaf tissues. Results indicated that the soil and epiphytic isolates did not showed any pathogenic characters in these plants and pectolytic activity was also not recorded.
- **Characterization of isolate HIP32** by cell morphology under electron microscope and by different cultural and biochemical tests we concluded that it was peritrichously flagellated, Gram-negative, and non-fluorescent, glucose utilization was positive, facultative anaerobic and non spore forming. Results indicated that the epiphytic isolate HIP32 belongs to the family *Enterobacteriaceae* as *Pantoea agglomerans* the aerogenic bio-group G1.
- **Characterization of soil isolate HIR225** showed that the soil isolate is non-fluorescent, non-pigmented peritrichously flagellated, Gram positive, and able to form endospores. Biochemical tests indicated that it belongs to the family *Bacillaceae* as *Bacillus subtilis.*

▪ **Quantitative analysis of pathogen survival in soil** by counting the numbers of their colony forming units (CFU) in accordance with time demonstrated the effectivity of the selected antagonistic strains HIP32 of *Pantoea agglomerans* and HIR225 of *Bacillus subtilis* against plant pathogenic species. The antagonistic strains were effectively reduced the *Xanthomonas vesicatoria* population and were also moderately effective against *Clavibacter michiganensis* subsp*. michiganensis.*

▪ **Fire blight disease reduction on pomaceous plant species** was developed to evaluate the utilization of *Pantoea agglomerans* and its mode of action on different host-pathogen combinations. During estimation of the capability of the antagonist to reduce fire blight disease symptoms in leaf-discs of different apple cultivars, treated leaves and fruits of apple, pear, quince, and flowers of cotoneaster. It was found that disease reduction in pre-treatment was higher (50-98%) although in post-treatment was effective in some cases (34-51%). On flowers of cotoneaster the same effectivity of the antagonist was observed as on other pomaceous plants inoculated with *Erwinia amylovora.* Disease reduction in leaves of cotoneaster and quince were more demonstrative than in apple and pear.

▪ **Leaf spot disease reduction on tomato and pepper seedlings** by the epiphytic antagonist HIP32 in greenhouse experiment using different combinations of treatments was clarified. Statistical analysis demonstrated that the disease reduction was more pronounced in pre-treated younger (6 leaves) tomato plants (45%) than in older (10,12 leaves) plants (30%). Disease reduction in pepper seedlings was also more pronounced in pre-treatment (40%)

An "additional" effect of the antagonist was observed in connection with improvement of pepper growth by measuring the leaf-size and leaf numbers of treated pepper plants. Results demonstrated that *Pantoea agglomerans* had not only a good effect on reduction of symptoms

(numbers of necrotic leaf spots) but it is also reduce the leaf fall as part of disease syndrome. Treated plants with the antagonist had improved good bearing and vigour with strong green color and larger leaf size. Results demonstrated that pre- and post-treatments had almost the same effect on the growth of pepper plants.

The protective effect of *Pantoea agglomerans* was demonstrated from the first day until 6th days of treatment

Results gave an indication that *Pantoea agglomerans* displayed strong effect in tomato and pepper plants as well as it was in pomaceous plants.

- **New scientific results**

Two new antagonistic strains were selected: from the phylloplane HIP32 and from the rhizosphere HIR225. They were identified as *Pantoea agglomerans* and *Bacillus subtilis* respectively. A wide *in vitro* antimicrobial effect was detected to most important bacterial plant pathogenic strains tested.

A reduction of disease severity was found by *Pantoea agglomerans* against fire blight disease of pomaceous plants and the leaf spot disease of tomato and pepper.

Pantoea agglomerans was tested *in vivo* as microbial inocula in apple tree foliage. According to its persistence, activity on disease reduction and epiphytic multiplication it is proposed as biocontrol agent against the above studied diseases.

Bacillus subtilis isolate could reduce *Xanthomonas vesicatoria* and *Clavibacter michiganensis* subsp. *michiganensis* populations in the soil.

4. CONCLUSIONS AND PROPOSALS

- In the phylloplane of apple trees as well as in the rhizosphere of a grown Hungarian and Libyan soils numerous bacterial species were found, which were effective against the plant pathogenic bacteria and fungi tested. Inhibition zones demonstrated that some isolates displayed wide spectrum and strong effectivity against these pathogens.

- According to the antagonistic tests, the epiphytic and the soil antagonistic isolates were selected on the basis of strongest and widest effects. Different morphological, cultural and biochemical tests suggested that the epiphytic isolate HIP32 is a member of *Enterobacteriaceae* and identified as *Pantoea agglomerans*. The antagonist *Erwinia herbicola* and *Enterobacter agglomerans* are the synymom. of *Pantoea agglomerans.*

- The soil isolate HIR225 was also identified as *Bacillus subtilis,* which is a member of family *Bacillaceae*. The saprophytic characters of the two selected strains were also confirmed.

- Antibacterial metabolite production was confirmed in culture filtrate (CF) of *Pantoea agglomerans* that causing inhibition zones on field of the pathogen and in the poisoned agar plate containing CF. The type of the media had a role in metabolite production by *Pantoea agglomerans.*

- Establishment and persistence of *Pantoea agglomerans* in its original host apple trees was found to be successful. Abundant multiplication was also confirmed when reisolated on selective medium.

- Reduction of fire blight disease by the antagonist was confirmed by different developed methods: as leaf discs, complete leaves, fruits and flowers of different pomaceous plants (apple, pear, quince and cotoneaster) treated with *Pantoea agglomerans* strain HIP32 before (pre-treatment) and after (post-treatment) with *Erwinia amylovora* strains. Disease reduction was more evident in pre-treatments than post-treatments with different effects.

- Analysis of visual disease symptoms demonstrated that the disease reduction was also more evident in pre-treated tomato and pepper seedlings

inoculated with *Xanthomonas vesicatoria*. On younger tomato plants disease reduction was more pronounced than the older one.

- The opinion that the antagonist was less effective in post-treatment is that the pathogen pre-colonized the intercellular spaces and starts to grow and multiply in favorable conditions 24 hr before the antagonistic strain application which could be difficult for the 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, which gave a chance for the antagonist to multiply and produce its metabolites.

Beside depression of symptoms and reduced leaf fall that demonstrated by leaf size and number measurements; more vigour, strong green color in each treatments were also observed. These additional effects of the antagonist on improvement of leaf size and leaf number may have an economical value.

- The protective effect of *Pantoea agglomerans* in pepper plants was evident from first day until 6th days treatments by *Xanthomonas vesicatoria* strain.

Both *Bacillus subtilis* and *Pantoea agglomerans* as biocontrol agents were demonstrated to reduce the colony forming units of the pathogens; *Xanthomonas vesicatoria* and *Clavibacter michiganensis* subsp. *michiganensis* in soil so they seem to be effective biocontrol agents. *Bacillus subtilis* is well known as biocontrol agent in soil conditions.

Epiphytic isolate HIP32 of *Pantoea agglomerans* was also found to be effective in the soil against soil borne and leaf pathogens. These results are evident thinking that *Pantoea agglomerans* is a soil inhabitant too

* A continued work is suggested with these two antagonists; *Pantoea agglomerans* and *Bacillus subtilis* in Libya where it have to be tested for adaptation to field conditions using different host-pathogen combinations.

* The selected biocontrol agents could be probably candidates in perspective biocontrol research programs.

* This study could be applied as a model for additional experiments in future for selection of other candidate biocontrol agents.

I hope that I have selected prospective biocontrol agents that can provide a protection to nature from over use of toxic chemicals that threatens human life and his environment.

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