EVALUATION OF SOME BACTERIAL ANTAGONISTS FOR BIOLOGICAL CONTROL OF FIRE BLIGHT DISEASE

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SUMMARY

Antagonistic activities of 120 epiphytic bacterial isolates against Erwinia amylovora were evaluated under laboratory and orchard conditions. Samples were collected at three stages of plant growth during 2009. The hosts were chosen from diseased and healthy pome fruit and stone fruit trees in Alborz, Isfahan and Neishabur regions of Iran. Two in vitro tests including antibiosis and immature fruit assay were performed to identify effective bacterial antagonists. Out of 21 strains, four representative strains encoded Kgh1, Abp2, E11 and E10 showed maximum growth inhibition, growth rate and stability in inhibition. Identification of four bacterial antagonists was confirmed by sequencing their 16S rDNA and performing common biochemical tests. These strains were identified as Pseudomonas fluorescens (E10), Pantoea agglomerans (Abp2), Pseudomonas putida (E11) and Serratia marcescens (Kgh1). Some complementary tests were then conducted to check their antagonistic activity against Erwinia amylovora on pear blossom. In all evaluation procedures, biocontrol effects of Pantoea agglomerans Abp2 and Pseudomonas fluorescens E10 were more promising than those afforded by Pseudomonas putida E11 and Serratia marcescens Kgh1. A one-year field experiment was also carried out on a partially susceptible domestic pear cultivar in an infested orchard in the Karaj region of Iran. The selected bacteria were applied twice at 20% and 80% of bloomings. The results showed 46.9% disease occurrence in control plants, while the antagonistic bacteria reduced the disease between 23 and 60%. In the orchard trial the most effective antagonist was P. agglomerans Abp2, while the least effective was S. marcescens Kgh1.

Key words: Erwinia amylovora, Pseudomonas fluorescens, P. putida, Serratia marcescens, Pantoea agglomerans, antagonism, biocontrol.

INTRODUCTION

Erwinia amylovora is the causal agent of fire blight, a devastating disease affecting species in the family Rosaceae (Abdollahi et al., 2004; Vanneste, 2000; van der Zwet and Keil, 1979). The disease was first observed in 1780 on apple trees in the Hudson Valley (New York, USA). Since then, it has been spread towards North America, some European countries, New Zealand, England and Mediterranean regions (Bonn and van der Zwet, 2000). E. amylovora usually causes severe economical damage to commercial production of pome fruit trees. In the United States, the annual damage of this disease has been estimated to be ca. 100 million US$ resulting from production decrease and cost of disease management (Norelli et al., 2003).

In Iran, the disease was first reported from pear trees of Karaj orchards in 1989 but now is found in many pomefruit-production areas of the country (Zakeri and Sharifnabi, 1991). The latest official reports estimate that the acreage of infested orchards (mostly apple and pear) in Tehran province amounts to ca. 20,000 ha.

Suppression of E. amylovora at full blooming stage on apple and pear is the key point in fire blight management (Schröth et al., 1974). Application of copper compounds during blooming have been recommended for disease control, but these chemicals usually cause phytotoxicity to blossoms and fruits (Loper et al., 1991; Tievoldale and Viveros, 1999). In addition, antibiotics such as streptomycin, which are very effective compounds, often induce antibiotic resistance in E. amylovora and are a potential threat to human health (Aldwinckle et al., 1998), so that the World Health Organization (WHO) has prohibited their use in plant protection (Ozakkan and Bora, 2004). Because of these limitations, it is inevitable to employ alternative control strategies, among which E. amylovora suppression on blossoms with the use of antagonistic bacteria has been considered as a viable alternative method for controlling fire blight disease (Beer et al., 1984).

Pseudomonas fluorescens and Pantoea agglomerans (syn. Erwinia herbicola) have extensively been studied as potential biocontrol agents of fire blight (Johnson et al., 1993; Johnson and Stockwell, 1998). P. fluorescens...
strain A506 that initially was selected for suppressing population of an ice nucleating strain of \( P. syringae \) on corn leaves (Lindow, 1985), is now available commercially (Blightban A506; Plant Health Technologies, USA) for the biological control of fire blight. This bacterial strain suppresses the growth of \( E. amylovora \) on blossoms by occupying the stigmas prior to colonization by the pathogen and utilizes nutrients important for its growth (Wilson and Lindow, 1993). In previous studies, a 40-60% (Johnson et al., 1993; Lindow et al., 1996) and up to 70% (Wilson and Lindow, 1993) disease reduction were observed following application of Blightban A506.

Some biological products, i.e. Blossom Bless and Bloom time developed from \( P. agglomerans \) strain P10c (Pusey, 1997) and \( P. agglomerans \) strain E325 (Vanneste et al., 2002), respectively, are now in the market. \( P. agglomerans \) strain Eh24 proved to be no less less effective than chemical treatments in restraining fire blight attacks (Ozaktan and Bora, 2004) and the use of \( P. fluorescens \) and \( P. agglomerans \) strain C9-1 or their derivates, resulted in blossom blight reduction from 43% to 83% (Sundin et al., 2008). \( P. agglomerans \) produces antibiotics that inhibit \( E. amylovora \) (Ishimaru et al., 1988).

Despite this considerable progress in the biological control of fire blight in the world, only a few studies have been carried out in Iran, thus, an investigation was conducted with the goal of evaluating some local bacterial antagonists against \( E. amylovora \) under laboratory and orchard conditions.

**MATERIALS AND METHODS**

**Isolation of \( E. amylovora \) strains.** During January-March 2009, infected shoots of apple, pear and quince were collected from Alborz, Isfahan and Neishabur provinces and transferred to the laboratory. The samples were washed and isolations were made on nutrient agar (NAS) kept for 48 h at 24ºC. The mucoid ples were washed and isolations were made on nutrient provinces and transferred to the laboratory. The samples were purified and stored in nutrient agar (NA) slants covered with mineral oil at 4ºC and, for prolonged preservation, the cultures were frozen at -80ºC or lyophilized (Mercier and Lindow, 2001; Harju et al., 2000).

**In vitro antibiosis test.** For identification of potential antagonists, each bacterial isolate was spot-inoculated in dual cultures on NA and KB culture media. After 48 h incubation at 25ºC, the \( E. amylovora \) strain \( K_9 \) suspension at concentration of \( 10^6 \) CFU/ml was sprayed uniformly on the surface of the inoculated Petri plates. This test was repeated 3 times and inhibition zones and their stability were determined (Lindow, 1988).

**Immature pear fruit assay.** Immature pear fruits of the partially susceptible domestic cultivar (cv. Shah Mieveh) were used for selection of effective antagonists (Beer and Rundle, 1983). A small well was made on the surface of each slice using a sterile borer. Ten \( \mu l \) of a fresh suspension of each bacterium at \( 10^8 \) CFU/ml concentration were then placed in each well. After 2 h incubation, 10 \( \mu l \) of \( E. amylovora \) strain \( K_9 \) suspension (\( 10^6 \) CFU/ml) were added to the same well. Petri plates were then incubated at 30ºC for 48 hr. In positive treatments, the growth of bacterial colonies was limited by potentially efficient antagonists.

**Detached pear blossom assay.** This experiment was conducted as a completely randomized design (CRD) with four treatments and nine replications for each treatment. Four efficient antagonists were selected based on antibiosis and immature fruit assays. Pear shoots (cv. Shah-Mieveh and Spadona) at the unopened blossom stage were collected and placed in flasks containing tap water for a week until the blooms opened. The newly opened flowers were then cut, their pedicels were immersed in sterile culture tubes containing 30 ml of 10% sucrose solution (Lawrence et al., 2009) and the flowers were inoculated by depositing 10 \( \mu l \) of each antagonist suspension in their center at the concentration of \( 10^6 \) CFU/ml. After 24 h incubation in a moist chamber at 25ºC with 90% relative humidity, an \( E. amylovora \) suspension (ca. \( 10^6 \) CFU/ml) was placed on the inoculation site. The treated blossoms were further incubated at 25ºC for 24 h. Potassium phosphate buffer, \( E. amylovora \) alone and antagonists alone were used as controls.

samples were treated with distilled water.

**Isolation, activity and identification of antagonistic bacteria.** Isolation of antagonistic bacteria. Bacterial strains were isolated from symptomless blossoms, leaves and shoots of pome and stone fruit trees collected from the above mentioned provinces and transferred to the laboratory. Samples were soaked in Erlenmeyer flasks containing 50 ml distilled water. After shaking for 30 min, 0.1 ml of each suspension was streaked on King’s B (KB) medium. All morphologically distinct colonies were purified and stored in nutrient agar (NA) slants covered at 4ºC and, for prolonged preservation, the cultures were frozen at -80ºC or lyophilized (Mercier and Lindow, 2001; Harju et al., 2000).

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The percentage of infected blossoms in control treatment showing necrosis of the stigma in cv. Shah-Mieveh and oozing and blackening of the stigma in cv. Spadona was determined six days post inoculation and the reduction percentage of infected blossoms was calculated.

**Attached pear blossoms assay.** This assay was performed as reported by Lelliott and Stead (1987) with slight modifications. The procedure was the same as described for detached pear blossom assay except that inoculations were made by spraying blossoms on the shoots. The percentage of infected blossoms was estimated in both cultivars by visually assessing the lack of necrosis (blackening) of stigma and ovary 5 days post inoculation.

**Biochemical tests for identification of effective bacterial antagonists.** For identification of effective bacterial antagonists, all standard biochemical tests were carried out, including Gram stain, oxidase, fluorescent pigment production on KB medium, levan production, pectinase (potato rot) and hypersensitive reaction (HR) on tobacco (Schaad et al., 2000). Each PCR reaction mixture contained 1.5x10^8 CFU/ml prepared in 10 mM potassium phosphate buffer (pH 7) were sprayed on the blossoms using a hand lever knapsack sprayer in the early morning in the absence of wind. Treatments were done twice at 20% and 80% of blooming stage. Plastic bags that covered blossoms and shoots after inoculation were removed on next day (Mercier and Lindow, 2001). There were two sets of control trees the first of which was sprayed only with potassium phosphate buffer whereas the other set remained untouched with no pruning nor spraying. Disease progress was monitored at 10 days intervals for a period of 2 months. The incidence of infection in both controls was determined using the following formula:

\[
\text{Fire blight disease (\%) = } \frac{(C-T)}{C} \times 100
\]

where \(C\) = Mean of infected shoots in control treatments and \(T\) = Mean of total treated shoots.

The percentage of disease reduction afforded by antagonists in comparison with control treatments was determined using the following formula:

\[
\text{Percent reduction of disease = } \frac{(C-T)}{C} \times 100
\]

**RESULTS**

**Identification and pathogenicity tests of the fire blight pathogen.** Ten typical *Erwinia amylovora* colonies were sub-cultured for further characterization. Three of them were identified based on phenotypic traits such as motility by peritrichous flagella, cell shape (rods), facultative anaerobiosis growth, levan production, lack of fluorescence on KB medium and negative response to Gram, catalase, oxidase, nitrate reduction, starch and esculin hydrolysis, indole, urease, \(H_2S\), growth at 39°C, acid production from salicin, sorbitol but not from melibiose, inositol and D-raffinose.

All bacterial isolates showed positive response to gelatin hydrolysis, grew at 5% NaCl, were hypersensitive in tobacco and failed to cause potato soft rot. They produced oozing on immature pear fruit slices and caused blossom blight. No symptoms were observed in the controls. In pathogenicity tests, no significant differences were observed among isolate, suggesting that there were not distinct differences in virulence. Therefore, only isolate K1 was used for further studies.

**Isolation, activity and identification of antagonistic bacteria.** In vitro evaluation of bacterial antagonists. Of
the 120 bacterial isolates, 32% were Gram-negative and ca. 50% showed inhibitory effects on *E. amylovora*. Fourteen isolates yielded inhibitory zones smaller than 5 mm, 15 isolates induced inhibitory zones of 5-10 mm and 21 isolates above 10 mm. It was noticed that growth inhibition zones produced on KB medium were 20% bigger than in NA medium (Table 1).

**Inhibitory effect of antagonists on immature fruit.** Out of 120 bacterial isolates, 29 isolates completely inhibited bacterial oozing on the surface of immature pear fruits, but eight of them, including DB3, N6, MaP3, E9, PP3, Pa2, FZ1 and N7, which showed inhibitory effects in immature pear fruit assay, failed to retard the growth of *E. amylovora* in culture media. Four bacterial isolates denoted Kgh1, Abp2, E11 and E10 were more effective in all tested conditions, thus were selected for further studies and field experiments.

**Results of biochemical tests for identification of effective bacterial antagonists.** Based on the results of standard biochemical tests (Table 2) four isolates denoted E10, Apb2, E11 and Kgh1 were identified as *Pseudomonas fluorescens*

![Fig. 1. Electrophoresis of 16S rDNA PCR product of antagonistic bacteria amplified with P0/P6 primer pair. Left to right: molecular marker 1 kb, blank, the next 4 lanes Pantoea agglomerans, Pseudomonas fluorescens, P. putida and Serratia marcescens, respectively.](image)

**Table 1.** Effect of bacterial antagonists in suppression of *Erwinia amylovora* on immature fruits and culture media.

<table>
<thead>
<tr>
<th>Inhibition zone <em>a</em> (mm)</th>
<th>Sampling location</th>
<th>Host characteristic</th>
<th>Isolate</th>
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<tr>
<td>IPFRA KB NA Province Species Isolate</td>
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<tr>
<td>- 16 14 Alborz Apple Meh5</td>
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<td>- 16 13.5 Alborz Plum Fsh3</td>
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<td>- 18 15 Alborz Peach Ksh1</td>
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<td>- 16 13 Alborz Peach Ksh2</td>
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<td>- 36 30 Alborz Plum Kgh1*</td>
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<td>- 12.5 11 Alborz Prunus sp. Kgt2</td>
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<tr>
<td>- 14 12 Alborz Peach Fh1</td>
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<td>- 11.5 10 Alborz Peach Pp</td>
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<td>- 14 12 Alborz Pearl Tp1</td>
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<td>- 14 23 Alborz Pearl Abp2*</td>
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<td>- 16 13 Alborz Pearl Kp2</td>
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<td>- 12 10.5 Alborz Pear Gp1</td>
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<td>- 14 11 Alborz Pearl Dp</td>
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<td>- 12 10 Alborz Apricot Kb</td>
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<td>- 13.5 11 Isfahan Apple E4</td>
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<td>- 19 16 Isfahan Quince E10*</td>
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<td>- 24 19 Isfahan Quince E11*</td>
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<td>- 0 0 Alborz Apricot FZ</td>
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<td>- 0 0 Neishabur Apple N6</td>
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<td>- 0 0 Alborz Apple MaP3</td>
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<td>- 0 0 Isfahan Quince E8</td>
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<td>- 0 0 Neishabur Apple N7</td>
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<td>- 0 0 Alborz Quince DB3</td>
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<td>- 0 0 Alborz Pear PP3</td>
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<td>- 0 0 Alborz Pear Pa2</td>
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*a Average of inhibition zones (mm) at three intervals.

*b Selected antagonists.*
(E10), Pantoea agglomerans (Abp2), Pseudomonas putida (E11) and Serratia marcescens (Kgh1), respectively.

Results of sequencing 16s rDNA for confirmation of antagonistic bacterial identity. PCR amplification using the universal primers P0 and P6 led to amplification of 1,500 bp fragments from all four antagonists. No clear band was observed in blank (Fig. 1). A 100% homology was found when the nucleotide sequences of these bacteria were compared with comparable sequences from GenBank. This was taken as confirmatory evidence that isolates E10, Apb2, E11 and Kgh1 are indeed \textit{P. fluorescens} (E10), \textit{P. agglomerans} (Abp2), \textit{P. putida} (E11) and \textit{S. marcescens} (Kgh1). Their sequences were deposited in GenBank under the accession numbers HQ420253.1, HQ420252.1, HQ840763.1 and HQ840765.1.

\textit{Detached pear blossom assay}. The antagonistic bacteria reduced disease incidence significantly compared to the control (P=0.01). \textit{P. fluorescens} E10 with 75.5\% blossom blight reduction on cv. Shah-Miveh (Fig. 2A), and \textit{P. agglomerans} Abp2 with 79\% blossom blight reduction on cv. Spadona, exhibited the most pronounced effects (Fig. 2B).

\begin{table}
\caption{Results of biochemical tests for the identification of the four effective bacterial antagonists.}
\begin{tabular}{lcccc}
\hline
Tests & E10 & E11 & Abp2 & Kgh1 \\
\hline
Gram stain & - & - & - & - \\
Anaerobic metabolism of glucose & - & - & + & + \\
Oxidase & + & + & - & + \\
Fluorescent pigment production on KB & + & - & - & - \\
Levan production & - & - & - & - \\
Pectinase & - & - & - & - \\
HR on tobacco & - & - & - & - \\
\hline
Isolate identity & \textit{Pseudomonas fluorescens} & \textit{P. putida} & \textit{Pantoea agglomerans} & \textit{Serratia marcescens} \\
\hline
\end{tabular}
\end{table}

Fig. 2. The mean percent of blossom blight reduction by antagonists compared to control (P $\leq$ 0.01) in Shah-Miveh cultivar (left) and Spadona cultivar (right). Treatments with similar letters are not statistically different at the 0.01 level according Duncan’s multiple range test. Pa, \textit{Pantoea agglomerans} Abp2; Psf, \textit{Pseudomonas fluorescens} E10; Psp, \textit{Pseudomonas putida} E11; Sm, \textit{Serratia marcescens} Kgh1.
Attached pear blossoms assay. The percentage of infected blossoms in control treatment (*E. amylovora*) was 100%. All selected putative antagonistic bacteria significantly reduced blossom infections at the 0.01 level. The biocontrol effect of *P. agglomerans* Abp2 and *P. fluorescens* E10 was more promising than that of *P. putida* E11 and *S. marcescens* Kgh1 (Fig. 3). Blossoms inoculated with bacterial antagonists did not show symptoms.

Field trial. Despite the unfavorable conditions for fire blight infections at blooming time in 2010, shoot blight occurred in the field trial (46.9% in untreated controls). The antagonist treatments significantly reduced disease symptoms from 60 to 23.2% compared to controls. The highest shoot blight reduction (60%) was obtained by *P. agglomerans* Abp2 and the least belonged to *S. marcescens* Kgh1 isolate with 23.24% (Fig. 4).

**DISCUSSION**

All tested bacterial antagonists produced inhibitory zones against *E. amylovora* in NA and KB culture media, but the inhibitory zones in KB were wider probably due to the higher growth rate of bacterial antagonists in this medium. Most of the isolates were potential antagonists as judged by suppression of bacterial exudation in immature pear fruits. There were exceptions, however, for strains DB3, N6, MaP3, E9, PP3, Pa2, FZ1 and N7 were effective in immature pear fruits but not in culture media. This may be attributed to other biocontrol mechanisms rather than antibiotic production, such as nonvolatile inhibitory metabolites, or competition, which has been well documented in previous studies (Temple et al., 2004; Stockwell et al., 2001; Wilson and Lindow, 1993). Although antibiosis is a key test for screening potential antagonists against plant pathogens, it is an unreliable factor for determining antagonistic activities of bacteria.
In this study, four bacterial species, *P. fluorescens* (E10), *P. putida* (E11), *P. agglomerans* (Abp2), and *S. marcescens* (Kgh1), proved to be the most effective antagonists in all *in vitro* and *in vivo* experiments. Their identification was achieved by biochemical tests (Schaad et al., 2001) and confirmed by sequencing their 16s rDNA (Picard et al., 2000). The activity of two of these species (*P. fluorescens* E10 and *P. agglomerans* Abp2) was comparable to what previously reported (Johnson and Stockwell, 1998; Vanneste and Yu, 1996; Wilson and Lindow, 1993). Likewise, also *P. fluorescens* and *P. agglomerans* had previously been shown to have a great potential as fire blight biocontrol agents through a mechanism based on antibiotic production (Meadow Anderson et al., 2004; Wright et al., 2000). Some strains of these latter species, however, are ice nucleating (Maki et al., 2000). The activity of two of these species (E10), proved to be the most effective antibiosis with *P. agglomerans*, demonstrated in experiments with fire blight. The results of this study may have practical application in fire blight management and open the way to the development of a product for practical applications will require more extensive field experiments under different environmental conditions.

**REFERENCES**


Biocontrol of fire blight disease


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