Efficacy of Some Probiotic Bacteria on *Erwinia amylovora* the Causal Agent of Fire Blight

M. Pourjafari[1], R. Saberi Riseh[1*], P. Khodaygan[1], A. Hosseinipour[2], and M. Moradi[3]

ABSTRACT

Fire blight, caused by *Erwinia amylovora* bacteria, is one of the well-known plant diseases in the world including major diseases of the fruit trees, especially apples and pears. In recent years, due to chemical nature of the pesticides damaging human health and environment, the importance of biological control is considered as alternative measure to manage plant diseases. To investigate the possibility of biological control of the pathogens, healthy foliar samples of apple, pear, and quince trees were collected from different regions of Kerman Province, Iran, and then, biocontrol activity of antagonist agents was evaluated under laboratory conditions. On the basis of the results, some of the antagonists could decrease the symptoms of the disease by 14.28-79.59%. Laboratory evaluation included investigating the disease severity in immature pears, biocontrol activity of antagonist agents in the plate assay, inhibitory siderophore production, biofilm formation capacity, drought stress tolerance, and silver nanoparticle synthesis capability, which showed that these antagonist agents could potentially control the disease. Among the 9 well-performed antagonistic isolates from apples and pears, Vr87 isolate was selected. The studies were confirmed by amplifying part of 16S rDNA region of the isolate, using specific primers. By comparing the results on the NCBI website, the selected isolate was identified as *Enterobacter* sp. genus. Among all selected isolates as successful factors in controlling fire blight pathogen, including isolated isolates and isolates in the collection of Vali-e-Asr University, *Bacillus subtilis* strain BsVRU1 in the Vali-e-Asr University collection, with 73.5% inhibition, had higher inhibitory power than the other antagonist isolates against the pathogen of fire blight disease.

Keywords: *Bacillus subtilis*, Biological control, Biosynthesis, Drought stress, Silver nanoparticle.

INTRODUCTION

Fire blight is one of the most important diseases of the fruit trees (Rosaceae family), especially apples and pears, throughout the world. Fire blight was first observed in Europe and UK in 1957, and now is present in around 43 countries (Van der Zwet, 2002). For the first time in Iran, *E. amylovora* was reported from pear trees in Karaj. Following years, the pathogen was found in many orchards in the provinces of Azarbaijan and Ghazvin (Rahnama and Mazarei, 2002). *Erwinia amylovora* belongs to the Enterobacteriaceae family, a pathogenic vascular, necrotic, highly pathogenic, and facultative anaerobic. Rosaceae, especially apple, quince, and pear are the most sensitive host. The symptoms of disease on the affected trees are visible as blight or canker on different plant organs such as blossom, shoot, branch, leaf, fruit and trunk. The most important symptoms that indicate disease progression are necrosis and secretion of leaking (bacterial ooze) on infected branches and fruits (Gross *et al.*, 2014).

---

[1] Department of Plant Protection, Faculty of Agriculture, Vali –e- Asr University of Rafsanjan, Islamic Republic of Iran.

Corresponding author; e-mail: r.saberi@vru.ac.ir

[2] Department of Plant Pathology, Faculty of Agriculture, Bahonar University of Kerman, Islamic Republic of Iran.

[3] Pistachio Research Center, Agricultural Research, Education and Extension Organization, Rafsanjan, Islamic Republic of Iran.

Also, polysaccharides such as levan and amylovoran, which are critical for pathogenicity and virulence on different host plants, were produced by *Erwinia amylovora*. These two polysaccharides also require and contribute to biofilm formation (Koczan *et al.* 2009). Three main classes of virulence genes that contribute to the ability of *E. amylovora* to cause fire blight disease have been found as ams (amylovoran synthesis), hrp (hypersensitive response and pathogenicity), and dsp (disease-specific) (Bugert and Geider, 1995).

To manage the disease, resistant or tolerant varieties, cultural, chemical, and biological methods may be applied either alone or integrated. Unfortunately, no definitive cure for infected plants has been identified until now, and the diseased tissues have to be eliminated by pruning. The emergence of fire blight pathogen resistance to the most effective antibiotic, streptomycin, and beneficial effects of other factors led to the complexity of fire blight management and prompted the need for more management of prevention. Preventing and reducing the incidence of the disease includes antibiotic use and biocontrol agents during flowering (Kamber *et al.* 2012). Recently, given the greater risk of chemical toxicants for humans and the environment, the importance of the application of biological control for the struggle against plant diseases has been revealed. In addition to environmental considerations, since there are no reliable methods to get rid of apple and pear fire blight disease, biological control of this disease has particular importance. Biological control of the disease has developed enormously over the past 20 years, and this progress has been enhanced by the selection of effective antagonistic strains, information on the ecology of epiphyte bacteria, and the high awareness of the mechanisms by which these strains are used in suppressing disease (Stockwell *et al.*, 2002). Nowadays, the use of healthy and low-risk methods that can be successful in the long-run is concerned by researchers. Among these methods, which have become widespread today, are the natural enemies of plant pathogenic agents for their biological control. Many studies have shown that a significant amount of organisms in the environment can affect the growth of pathogens and the progression of the disease caused by them. The effects of other organisms on the progression of plant diseases in natural conditions has always been a factor in increasing the studies in this field. *Pseudomonas fluorescens* and *Pantoea agglomerans* have been extensively investigated for their abilities to control the disease caused by *Erwinia amylovora*. However, researchers have also evaluated new antagonists such as *E. amylovora* non-malignant strains, yeasts, gram-positive bacteria, and a mixture of specific bacteriophages for *E.amylovora*, for biocontrol of fire blight. Although these agents have shown hope in laboratory tests, they have not been tested under garden conditions as much as *P. fluorescens* and *P.agglomerans* (Johnson *et al.*, 2000). The *Pseudomonas fluorescens* (A506) was first isolated in California in 1984 from pears and has been made commercially available for biological control of fire blight since 1996. The antagonist *Pantoea agglomerans* (EhC9-1) was removed from apple in Michigan in 1988, and attempts to commercialize it began in 1997 to control fire blight. Antagonistic bacteria *Pseudomonas flourescens* (A506), *Pantoea agglomerans* (C9-1) and *Pantoea agglomerans* (E325) and bacteria formulated with *Bacillus subtilis* QST 713 and Streptomycin have been more effective and the percentage of reduction of blossom contamination varied from 59.5 to 67.7 (Koczan, 2009).

In the case of fire blight disease, the hypothesis that this study was carried out around this subject is the presence of antagonistic bacteria with acceptable biocontrol potency against the pathogen agent in vitro condition. Therefore, in the present study, the epiphytic and native bacteria were considered to be suitable factors for effective control of the bacterial agent of apple and pear fire blight disease.

MATERIALS AND METHODS

Biocontrol Agents

Sampling was conducted in infected orchards from the healthy tissues of apple, pear, peach and apricot in different agro-ecological regions of Kerman Province during spring, summer, and fall. Isolation of bacteria and yeasts from leaves and fruits was done according to Sakthivel and Mew (1991). Bacterial isolates were grouped

based on the color, appearance, density or frequency, Gram stain, and fluorescence color production that was used by King-B medium and Hypersensitivity Reaction (HR) on geranium plants (Suslow *et al.*, 1982; Schaad *et al.*, 2001; Klement *et al.*, 1964).

Pathogen

Erwinia amylovora strain Ea.42 (Department of Plant Pathology, Shahid Bahonar University of Kerman) was applied in all experiments. For daily work, *E. amylovora* was cultured on Nutrient Agar (NA) and kept at 4°C in sterile distilled water during the work. The pathogenicity of this strain on the immature pear fruits was assessed as described by Goszczynska *et al.* (2000).

Screening of Antagonist Agents

Hypersensitivity reaction test

The ability of the isolates by suspending 24-hour old bacteria to produce hypersensitivity on geranium leaves was evaluated within 24-48 hours after inoculations (Schaad *et al.*, 2001).

Interaction between antagonists and pathogen (Biocontrol assays on immature pear fruit)

Biocontrol activity of antagonistic agents on immature pear fruit was investigated using Ishimaru *et al.* method (1988). Immature pear fruits were collected in spring and stored at 4°C for a short period. Immature pear fruits with sterilized surface were inoculated with 10 μL (10^7 CFU mL^{-1}) of selected antagonist agents at a triangular shear with low depth at the cutting site. Afterward, 10 μL of *E. amylovora* strain Ea.42 (10^7 CFU mL^{-1}) was inoculated in the same place. Immature pear fruits were incubated for 7 days at 27°C and darkness. The severity of the disease was rated using 5 scales indices as described by Ishimaru *et al.* (1988). The experiments were carried out in three replicates for each antagonist isolates.

Plate assay

In order to investigate the inhibitory effect of antagonistic agents on the fire blight agent, a test was conducted to study the inhibitory zone diameter at the Petri dish level. For this purpose, a concentration of 10^9 CFU mL^{-1} was prepared from the pathogen bacteria. The bacterial suspension inhibited the disease on the NA culture medium uniformly. After drying the culture medium, antagonistic bacteria was placed on the surface of the medium. For each antagonist, three replicates were considered. After 72 hours of incubation at 28°C, size of inhibition zone was measured (Petruta *et al.*, 2008).

Assessment of Potential Ability of Antagonist Agents

Siderophore production

To measure the amount of siderophore of the antagonist agents, 100 μL of 24-hour culture of bacteria in a succinic liquid medium was transferred to 100 mL Erlenmeyer containing 40 mL of fresh succinate culture medium. The culture mediums were stored on a shaker for 40 hours at 27°C and 120 rpm. The bacterial cells were centrifuged in 1,000×g for 10 minutes. After removing the bacterial sediment, the absorbance of the supernatant at 400 nm wavelength was read by Spectrophotometer. The data obtained using the following formula was converted to mol per liter (Meyer and Abdallah, 1978).

A = εBC

Where, A=Absorption rate; ε= Molar absorption coefficient (25.5); B= Diagonal cuvette, C (mol L^{-1})= Concentration of matter.

Biofilm Assay

The first suspension of antagonist agents to measure and compare biofilm formation quantitatively was prepared in LB medium (with acidity equal to PH= 7). Then, 200 μL of suspension (with concentration of 10^4 cells

mL^{-1}) was added to the wells of Eliza Plate. The control wells were also added to the same sterile culture medium without bacteria. Plates were incubated at 28°C for 48 hours. In the next step, the medium containing the bacteria grown out of the wells was removed and each plate was washed three times with PBS buffer. In order to fix the bacteria bound to the wall and bottom of the well, about 200 μL of alcohol 96 percent (Ethanol) were added to each well. After 15 minutes, the contents of the wells were evacuated and the plates were placed in lab conditions until they were completely dry. Then, in each well 250 μL of 0.01% crystal violet solution was added and stored for 30 minutes at room temperature. The washing steps were repeated with the buffer three times, and finally, the absorbance of each well was read by ELISA reader device at 590 nm in length. This test was performed in three replications. Data analysis was analyzed by SAS software using the least significant difference (P< 0.05) (Giobbe *et al*., 2007).

Synthesis of Silver Nanoparticles

This experiment was conducted to evaluate the synthesis of silver nanoparticles by Kalishwaralal *et al.* (2008). Finally, the comparison was made for the production of silver nanoparticles. In the first step, changing color was considered, then, the absorption rate was measured by a spectrophotometer apparatus at 420 nm. Finally, to determine the size of the nanoparticle synthesized by the DLS (Dynamic Light Scattering) device of the Nanophox 90-246V model, the samples were sent to Shahid Beheshti University in Tehran (Kalishwaralal *et al*., 2008). The experiment was performed with three replications and analysis of data was done by ANOVA and mean comparison using SAS statistical software.

In order to investigate the effect of silver nanoparticles on bacterial growth inhibitory activity and determine the effective concentration of this substance on *Erwinia amylovora*, a causative agent of fire blight of apple and pear, a laboratory experiment was conducted. We used a completely randomized design and different doses of silver nanoparticles, which included control treatments (without silver nanoparticles) and other treatments including concentration of 10, 30, 60 ppm, in 3 replications. After preparing KB culture medium and autoclave medium at 121°C, when the ambient temperature reached about 45°C, 60 mL culture medium was poured into Erlene. Then, the specified amount of silver nanoparticles produced in the Department of Chemistry, Vali-e-Asr University of Rafsanjan, was added to it and was shaken slowly so that the nanoparticles of silver were mixed well with the culture medium. Then it was poured into 3 Petri and allowed to cool, this procedure was repeated for all doses. This procedure was repeated for all doses. A 24-hour culture of *E.amylovora* strain Ea.42 bacterium was prepared and 50 μL of it was cultured in culture medium containing different concentrations of silver nanoparticles. Control treatments were prepared for each bacterium using non-nanoparticle culture media. All Petri dishes were stored at 28°C in the dark for 48 hours and bacterial colonies (CFU) were counted in different doses of silver nanoparticles.

Drought Stress Tolerance

In order to evaluate the tolerance of antagonist agents to different levels of drought, Nutrient Broth (NB) medium containing 0, 203.362, 298.587, 438.440 and 548.838 g of polyethylene glycol 6,000 per kg of NB culture medium was used. The experiment was conducted using the method developed by Michel and Kaufman (1973). Three replications of the NB medium containing different amounts of polyethylene glycol were also provided as controls to determine the optical density of this medium in the above conditions and their optical density OD was read (Michel and Kaufman. 1973).

Data Analysis

Data analysis was performed using SAS software. The mean comparison was done at 5% probability level. Finally, using EXCEL and WORD software, graphs, tables and

Table 1. Primers used in this study to amplify the part of the *16S rDNA* gene of the bacterial genome.

Primer	Sequences	Connection temperature(°C)	Length (base pair)	Reference
63f	*CAGGCCTAACACATGCAAGTC*	65	1500	(Marchesi *et al.* 1998)
1387r	*GGGCGGWGTGTACAAGGC*			

information displays were used to compare them.

Identification

Molecular Identification

In order to assure the identity of antagonist bacteria, after extraction of bacterial DNA, specific primers were used in the Polymerase Chain Reaction (PCR). The nucleic acid of the selected bacteria was extracted with moderate phenol-chloroform method (Ausubel *et al.*, 1988). The quality and quantity of extracted DNA were also assessed by electrophoresis and spectrophotometry (Sambrook *et al.*, 1989). For sequencing of bacteria, two primers 63f and 1387r with the following sequences (Table 1) were used in the PCR reaction to amplify 16S rDNA region. The analysis of gene sequence was carried out using the BLAST program with the information contained in the Gene Bank's National Center for Biotechnology (NCBI) database (Stenstrom *et al.*, 1990).

Study of Phenotypic and Biochemical Properties

In addition to the tests used in the early grouping of isolated antagonist isolates, some phenotypic and biochemical properties were also studied to identify one of the selected isolates. Selected isolate based on the production of specific pigments on culture medium such as Nutrient Agar(NA), King-B(KB) and Eosin Methylene Blue(EMB), Yeast Extract-Dextrose-Calcium carbonate(YDC) based on oxidase test (Kovacs, 1956), based on aerobic/anaerobic growth (O/F) using glucose, or peptone

(Hugh and Leifson, 1953), the production of indole from tryptophan (Lelliott and Stead, 1987), the test for arginine dihydrolase (Thornley, 1960), production of lesitinase (Lelliot *et al*., 1966), production of levan in a Nutrient Agar Sucrose(NAS) medium medium (NA environment with 5 sucrose percentage), gelatin hydrolysis, starch hydrolysis, the ability to grow isolates at 41°C, the use of some carbon sources (carbohydrates) and the production of acid from some sugars in the Ayr, Methyl Red, Catalase test, Gram stain, and hydrolysis of ascolin. The method of Schaad *et al.* (2001) was carried out and its results were investigated.

RESULTS

Screening of Antagonistic Isolates and Pathogenic Bacteria

The branches and leaf samples were collected from various trees including apple, quince, pear, peach and apricot around different regions of Kerman and, generally, 134 bacteria were isolated. The isolates without necrosis and water-soaked symptoms on geranium leaf were selected as isolates with antagonistic potential and the isolates with positive hypersensitivity reaction on geranium leaves were eliminated. As a result, 49 bacteria isolates with negative hypersensitivity response were determined. Additionally, 6 antagonistic strains existing in Plant Pathology Department Collection were used in this study and other tests. Pathogenic bacterium *E.amylovora* with Ea.42 code was obtained from plant pathology department of Shahid Bahonar University of Kerman (Table 2).

Table 2. Bacteria used in experiments.

Strain	Region	Host	Strain	Region	Host	Strain	Region	Host
Vr1	Sarcheshmah	Apple	Vr73	Bardsir	Apple	Vr169	Shahrbabak	Quince
Vr4	Sarcheshmah	Apple	Vr74	Bardsir	Apple	Vr170	Shahrba bak	Quince
Vr5	Sarcheshmah	Apple	Vr79	Bardsir	Apple	Vr195	Kerman	Apricot
Vr6	Sarcheshmah	Apple	Vr81	Bardsir	Apple	Vr202	Kerman	Apricot
Vr7	Sarcheshmah	Pear	Vr82	Bardsir	Apple	Vr204	Kerman	Apple
Vr8	Sarcheshmah	Pear	Vr84	Bardsir	Apple	Vr205	Kerman	Apple
Vr9	Sarcheshmah	Apple	Vr85	Bardsir	Apple	Vr206	Kerman	Apple
Vr20	Shahrbabak	Quince	Vr87	Bardsir	Apple	Vr210	Kerman	Apple
Vr22	Shahrbabak	Quince	Vr98	Bardsir	Peach	Vr215	Kerman	Apple
Vr23	Shahrbabak	Quince	Vr102	Bardsir	Pear	Vr226	Kerman	Apple
Vr31	Shahrbabak	Quince	Vr113	Neyshabur	Apple	Vr228	Kerman	Apple
Vr38	Shahrbabak	Quince	Vr114	Neyshabur	Apple	*Bacillu subtilis* BsVRU	Pariz	Pear
Vr50	Shahrbabak	Quince	Vr123	Rafsanjan	Apple	*Bacillus subtilis* BsVRU1	Sirjan	Pistachio
Vr52	Shahrbabak	Quince	Vr145	Shahrbabak	Pear	*Bacillus subtilis* Bs96	Rafsanjan	Pistachio
Vr53	Shahrbabak	Quince	Vr148	Shahrbabak	Pear	*Pseudomonas fluorescens* T17-4	Tehran	Plantago
Vr54	Shahrbabak	Quince	Vr150	Shahrbabak	Pear	*Pseudomonas fluorescens* Vupf5	North of Iran	Peach
Vr55	Shahrbabak	Quince	Vr158	Shahrbabak	Pear	*Pseudomonas fluorescens* CHA0	Switzerland	Tobacco
Vr56	Shahrbabak	Quince	Vr162	Shahrbabak	Quince			
Vr64	Shahrbabak	Quince	Vr166	Shahrbabak	Quince			

Evaluation of the Antagonistic Potential of Isolates

Potential isolates (HR-) for reducing disease intensity of *E.amylovora* on unripe pear fruit and petri dish were evaluated. Suppression level on unripe pear fruit varied from 14.28% to 79.59% and the difference between bacterial isolates was significant. Among the investigated isolates, the highest amount of suppression on unripe pear fruit belonged to Vr5, Vr50, Vr54, Vr87, *B. subtilis* strain BsVRU1, *B. subtilis* strain Bs96, *B. subtilis* strain BsVRU and *P. fluorescens* strain T17-4 that led to disease intensity reduction of 67.3, 73.5, 71.4, 77.6, 73.5, 79.6, 79.6 and 77.6%, respectively. Also, the least suppression level was observed by Vr228 isolates, which caused only 14.28% disease intensity. Potential of the best 15 isolates in unripe pear fruit test was studied on petri dish. Four isolates had the ability to produce the largest suppression halo zone diameter around the disease agent colonies on the plates.

However, none of these four isolates showed hypersensitivity reaction on geranium leaves. Among the 15 isolates, *P. fluorescens* strain T17-4, Vr6, *B. subtilis* strain BsVRU1 and *P. fluorescens* strain VUPF5 isolates produced the most suppression level. The most and the least percentage of suppression to pathogen on petri dish was observed in *B. subtilis* strain BsVRU1 and *P. fluorescens* strain VUPF5 isolates, respectively (Tables 3 and 4) (Figures 1 and 2).

Assessment of the Potential Ability of Antagonist Agents

Siderophore production by spectrophotometry technique

The results obtained for 15 isolates in 400 nm using formula A= εBC was converted to

Table 3. The effect of antagonistic agents on the severity of the disease caused by *Erwinia amylovora* Ea.42 in immature pear fruit.

Treatment	Mean disease-severity rating	Treatment	Mean disease-severity rating	Treatment	Mean disease-severity rating
Water; Ea.42	4.9 a	Vr64; Ea.42	3.5 g	Vr166; Ea.42	1.7 u
Vr1; Ea.42	2 r	Vr73; Ea.42	3 l	Vr169; Ea.42	2 s
Vr4; Ea.42	3.9 c	Vr74; Ea.42	2.9 m	Vr170; Ea.42	3.1 j
Vr5; Ea.42	1.6 v	Vr79; Ea.42	2.5 p	Vr195; Ea.42	3.7 e
Vr6; Ea.42	1.9 t	Vr81; Ea.42	3.7 e	Vr202; Ea.42	3 l
Vr7; Ea.42	2.5 p	Vr82; Ea.42	4 b	Vr204; Ea.42	3.3 i
Vr8; Ea.42	2 r	Vr84; Ea.42	2.9 m	Vr205; Ea.42	2.1 r
Vr9; Ea.42	3 k	Vr85; Ea.42	3.2 j	Vr206; Ea.42	2.3 q
Vr20; Ea.42	2.7 o	Vr87; Ea.42	1.1 y	Vr210; Ea.42	2.9 m
Vr22; Ea.42	3.8 d	Vr98; Ea.42	1.7 u	Vr215; Ea.42	3.4 h
Vr23; Ea.42	3.1 k	Vr102; Ea.42	3 l	Vr226; Ea.42	2.9 m
Vr31; Ea.42	3.2 j	Vr113; Ea.42	3 l	Vr228; Ea.42	4.2 b
Vr38; Ea.42	3.2 j	Vr114; Ea.42	3.1 k	BsVRU; Ea.42	1 z
Vr50; Ea.42	1.3 x	Vr123; Ea.42	3.6 f	BsVRU1; Ea.42	1.3 x
Vr52; Ea.42	2 r	Vr145; Ea.42	2 s	Bs96; Ea.42	1.1 y
Vr53; Ea.42	2.8 n	Vr148; Ea.42	3.8 d	T17-4; Ea.42	1 z
Vr54; Ea.42	1.4 w	Vr150; Ea.42	1.7 u	VUPF5; Ea.42	1.9 t
Vr55; Ea.42	2.7 o	Vr158; Ea.42	3.3 i	CHA0; Ea.42	1.9 t
Vr56; Ea.42	1.9 t	Vr162; Ea.42	3.4 h	Water(C⁻)	1 z

Table 4. The amount of inhibition of the antagonists against *E. amylovora* strain Ea.42.

Mean inhibition zones(mm).	Isolate
1.5	*B. subtilis* strain BsVRU1
0.83	Vr6
0.73	*P. fluorescens* strain T17-4
0.1	*P. fluorescens* strain VUPF5

Figure 1. Effect of the treatment of wounds of immature pears with antagonist agents on the intensity of infections after inoculation with *Erwinia amylovora* in five different index. BsVRU1: *Bacillus subtilis* BsVRU1 (Index: 1.3), Vr52 (Index: 2), Vr9(Index: 3) and Vr228 (Index: 4.2), and *Erwinia amylovora* Ea.42 (Index: 4.9).
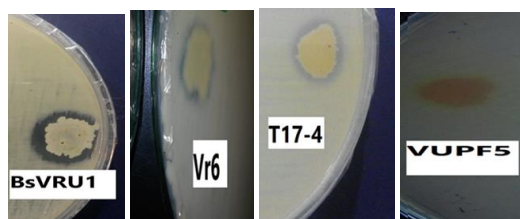
Figure 2. Inhibition of fire blight pathogen growth by *Bacillus subtilis* strain BsVRU1, *Pseudomonas fluorescens* strain T17-4, *Pseudomonas fluorescens* strain VUPF5, and Vr6 on the NA medium.

mol per lite and then converted to µmol L⁻¹.
Among the 15 tested isolates, Vr50 and Vr150
isolates produced the most by 2300 µmol L⁻¹
pyoverdine and showed significant difference
with other isolates statistically. Vr98, Vr56, and
Vr87 isolates produced 2,000, 1,900 and 1,900
µmol L⁻¹. Also, the least amount of pyoverdine
belonged to *B. subtilis* strain BsVRU1, *B. subtilis*
strain Bs96, and *P. fluorescens* strain T17-4,
whose pyoverdine production level in 3 strains
was 100 µmol L⁻¹ (Figure 3).

Biofilm formation

In investigating the biofilm producing ability,
absorption level at 590 nm was high, indicating
high-biofilm producing ability of the isolates.
Analysis of the biofilm formation proved that
there were significant differences between
bacterial isolates and the negative control. Also,
Bacillus subtilis strain Bs96 and Vr5 and Vr87
isolates determined light absorption with 0.23,
0.14 and 0.12, respectively. The results showed
that the isolates had high potential in biofilm
formation (Figure 4).

Drought Tolerance Level of Isolates

Stress tolerance level of 15 selected isolates to
drought proved that with increasing polyethylene
glycol (PEG) concentration, isolates growth
decreased significantly. Isolates growth rate at
level -5, -10, -20 and -30 bars decreased from 1.6
to 17.8%, 2.6 to 27.3%, 3.4 to 35.6%, 5.2 to
50.7%, respectively, compared to the control
without PEG. In addition, growth rate of Vr6,
Vr166 and *B.subtilis* strain BsVRU1 isolates was
insignificant at -20 and -30 level. According to
the results, Vr87 isolate showed the most
drought-tolerance in various osmotic potentials
with light absorption of 1.29, 1.34, 1.38, 1.42 and
1.51, (Optical density=OD), respectively, for -30,
-20, -10 and -5 bars (Table 5).

Biosynthesis of Silver Nanoparticles in Antagonistic Isolates

To assess silver nanoparticles production,
isolates potential in nitrate solution change of

color from yellow to brown was used. Among
the tested isolates, *B. subtilis* strain BsVRU
showed the most color change compared to
others (Figure 5). Maximum absorbance bands
were concentrated at 420 nm under UV-Vis
spectra of antagonistic isolates, which
confirmed the existence of silver
nanoparticles. Based on mean's comparison of
isolates light absorbance at 420 nm, the results
of silver nanoparticles biosynthesis test
showed that *B. subtilis* strain BsVRU isolate
included the maximum amount (significant at
1% level) of silver nanoparticles biosynthesis
in comparison to the control and other isolates
(Figure 6).

Amount of silver nanoparticles synthesized
by *B. subtilis* strain BsVRU was evaluated at
700 nm using DLS (Figure 7). Also, the results
of using various doses of industrial silver
nanoparticles on *E. amylovora* Ea.42
population confirmed that with increasing
nanoparticles, bacterial colony numbers
decreased, and for 0, 10, and 30 ppm were
216.7, 673.3, 1,766.7 and 2,083.3,
respectively. The obtained data proved that
nanoparticles decreased the bacterial
population between 15.2 to 89.6 (Figure 8).

Determination of Antagonists

Among the antagonistic strains that were
investigated from various orchards about
suppression potential to fire blight pathogen,
Vr87 isolate was selected for phenotypic,
biochemical and molecular characteristics and
orchard tests and the next tests. The main
cause for the selection was successful
biocontrol in unripe pear fruit test and high
potential in biofilm producing compared to
other isolates. Additionally, for exact and best
determination of Vr87 isolate, bacteriological
standard techniques were used and the results
are summarized in Table 6. On the basis of
evaluating phenotypic, biochemical and
genotypic characteristics of the selected
isolates in laboratory, researches identified that
Vr87 isolate was the most similar to
Enterobacter sp. by 90%. However,
Enterobacter species are motile bacteria,
facultative anaerobic, Gram-negative and rod-
shaped forms of which are saprophyte in
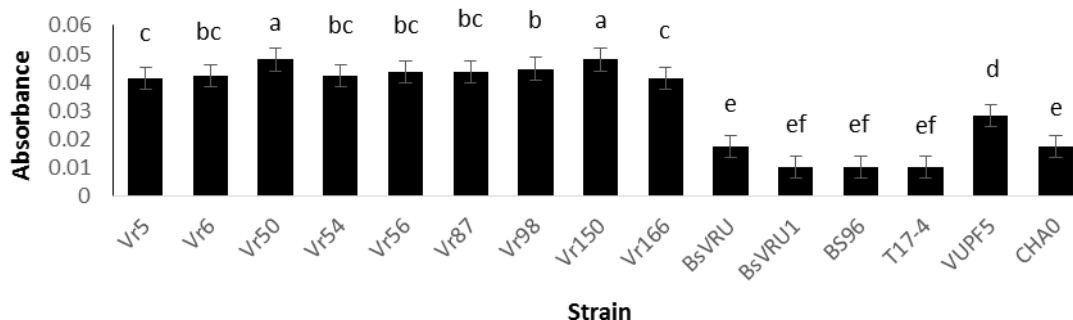
Figure 3. Production of siderophores by the spectrophotometric method in 400 nm. In succinate culture medium. BsVRU: *Bacillus subtilis* strain BsVRU; BsVRU1: *Bacillus subtilis* strain BsVRU1; Bs96: *Bacillus subtilis* strain Bs96; T17-4: *Pseudomonas fluorescens* strain T17-4; VUPF5: *Pseudomonas fluorescens* strain VUPF5; and CHA0: *Pseudomonas fluorescens* strain CHA0.
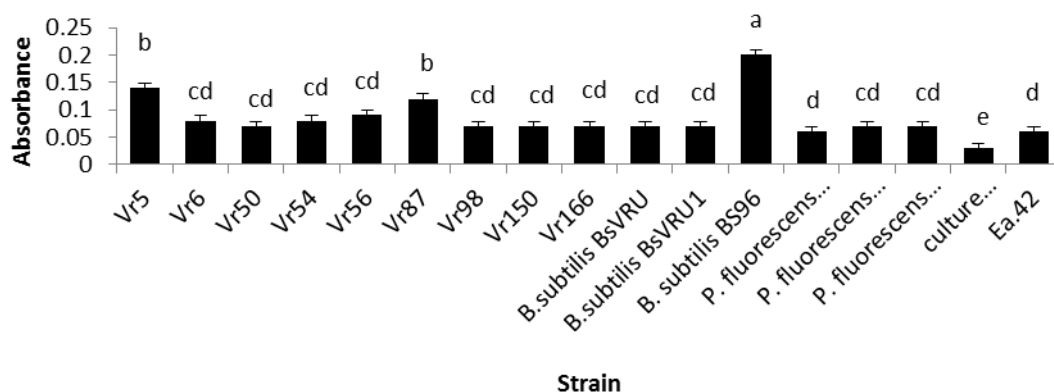

Figure 4. Biofilm production capability graph by antagonist agents based on the absorbance of light at 600 nm (Biofilm Plate) data. The data is an average of three replicates per antagonist. C-: Negative control.

Table 5. Growth status (OD 600 nm) of isolates at different PEG levels: The amount of PGE g kg^{-1} culture medium.

| Strain	0 Bar (0)	-5 Bars (203/362)	-10 Bars (298/587)	-20 Bars (438/40)	-30 Bars (548/838)
Vr5	OD= 1/32f-l	1/27j-r	1/24n-t	1/21r-v	1/17t-y
Vr6	OD= 0/82de	0/77ef	0/74fg	0/70f-h	0/68gh
Vr50	OD= 1/16u-y	1/13w-a	1/13w-a	1/12w-a	1/10y-b
Vr54	OD= 1/30h-o	1/27k-r	1/21q-v	1/18b-f	1/09z-b
Vr56	OD= 1/41b-e	1/37c-h	1/27j-r	1/25m-s	1/21r-v
Vr87	OD= 1/51a	1/42bc	1/38b-g	1/34f-j	1/29i-p
Vr98	OD= 1/31g-m	1/29i-p	1/27j-r	1/26l-r	1/23o-t
Vr150	OD= 1/43bc	1/35d-i	1/28i-p	1/22p-v	1/18s-w
Vr166	OD= 1/18s-w	0/97c	0/87d	0/76ef	0/74d
B. subtilis BsVRU	OD= 1/45ab	1/42bc	1/36c-h	1/33f-k	1/21r-v
B. subtilis BsVRU1	OD= 0/77ef	0/65h	0/56i	0/52i	0/38j
B. subtilis Bs96	OD= 1/41b-e	1/34e-j	1/23p-u	1/18s-x	1/11w-a
P. fluorescens T17-4	OD= 1/42b-d	1/37c-h	1/31g-m	1/26k-r	1/22p-v
P. fluorescens CHA0	OD= 1/15v-z	1/11x-b	1/08z-b	1/07ab	1/04bc
P. fluorescens VUPF5	OD= 1/36c-h	1/31h-n	1/27j-r	1/25l-r	1/23p-u
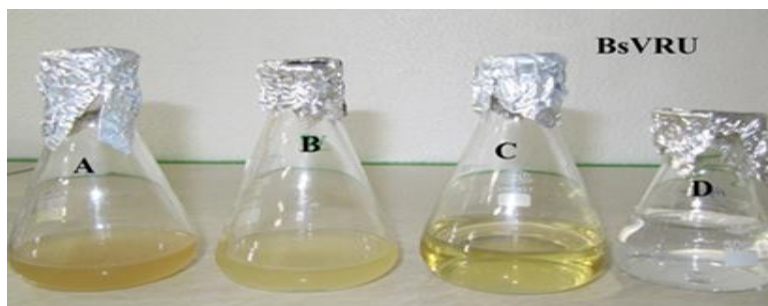
Figure 5. Biosynthesis of silver nanoparticles by *Bacillus subtilis* strain BsVRU. Erlenmeyer A: Content of bacterial killed cells with silver nitrate solution after 24 hours (darker color change); Erlenmeyer B: Content of cells that were killed without silver nitrate solution after 24 hours (no color change); Erlenmeyer C: Content of NB medium plus Silver nitrate solution without bacteria (no color change); and Erlenmeyer D: Contains silver nitrate solution without bacteria (no color change)
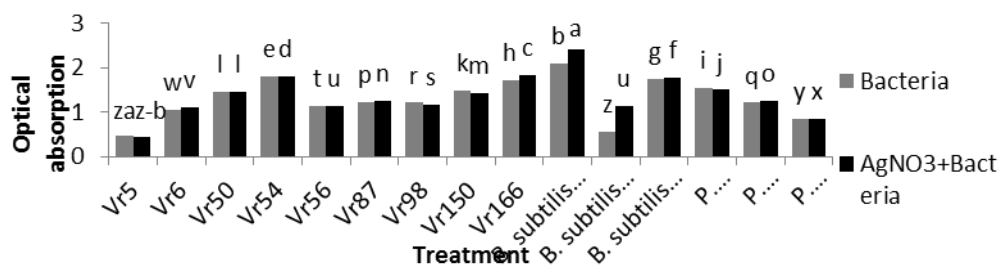
Figure 6. The graph of the synthesis of silver nanoparticles by antagonistic bacteria at 420 nm.
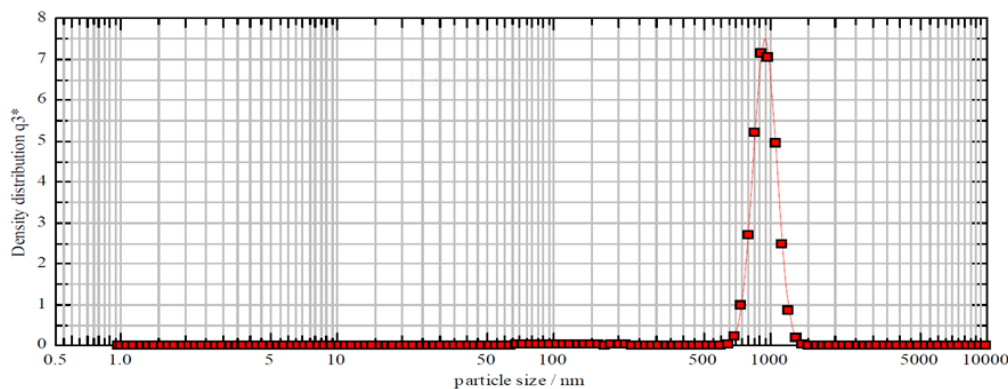
Figure 7. DLS Diagram prepared from nanoparticles of synthesized silver from *B. subtilis* strain BsVRU.
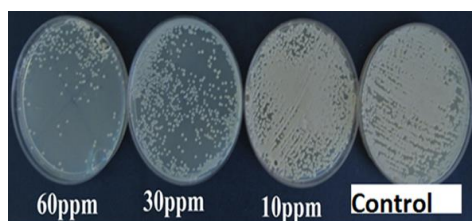

Figure 8. Effect of different concentrations of silver nanoparticles on growth of *E. amylovora* Ea.42 in comparison with the control.

Table 6. The phenotypic and biochemical characteristics of bacterial isolates Vr87

Test	Result	Use of carbohydrate	Result
Oxidase	-	Adonitol	-
Catalase	-	Sorbose	-
Gram stain	-	Sorbitol	+
Formation of Levan	+	Rhamnose	+
Oxidative/Fermentation (O/F) test	Facultatively anaerobic	Xylose	+
The color on NA	Light cream	Glycerol	+
The fluorescent pigment on King's medium B	Light cream	Ribose	+
The color on YDC	White	Glucose	+
Metallic paint production on EMB medium	+	Sacarose	+
Indole	-	Arabinose	+
the production of lesitinase	+	Fructose	+
Methyl red	+	Mannose	+
arginine dihydrolase	-	Lactose	+
Growth at 41˚C	+	Inositol	-
hydrolysis of ascolin	+	Dulcitol	-
gelatin hydrolysis	+	Mannitol	+
starch hydrolysis	+		

environment. For molecular determination of Vr87 isolate, after isolate DNA extraction, 16S rDNA sequences were amplified. Then, PCR products were electrophoresed on agarose gel (1%). Also, 16S rDNA sequences amplification by primers proved that selected isolate Vr87 with 1,500 bp long belongs to genus *Enterobacter* sp. (Figure 9).
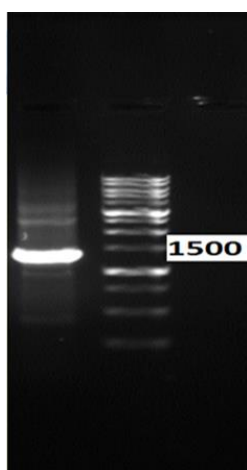
Figure 9. PCR results of isolate Vr87 for fragment proliferation 16S rDNA with a length of 1,500 bp with pairs of primers 63f and 1387r.

DISCUSSION

Fire blight is considered as a serious threat in Iranian pear orchards, restricting pear's production in some years. Recently, the disease has emerged as one of the most important plant pathogens in Iran and many other countries. Biological control of fire blight has developed during the last 20 years and has increased by selecting effective antagonistic strains, collection of data from ecology of epiphytic bacteria, and knowing the mechanisms that these strains apply to inhibit the disease.

In this research, the impact of multiple antagonistic bacteria was investigated in the presence of *E. amylovora* pathogen as a pear fire blight agent by suppression of halo formation test and unripe pear fruit test. Among the 55 antagonistic isolates, 15 isolates had minimum degree of disease severity in unripe pear fruit test and showed acceptable suppression against fire blight agent. Among these isolates, *B. subtilis* strain BsVRU1 emerged with considerable results in growth suppression of pathogen to others. In other words, the most suppression of halo diameter in petri dish was obtained using suppression

halo test. Then, *P. fluorescens* strain T17-4 showed the best suppression impact on pathogen growth in the laboratory. Evaluation of the impacts of antagonistic isolates in unripe pear test determined that *Enterobacter* sp. Vr87, *B. subtilis* strain BsVRU1, *B.subtilis* strain BsVRU, *B. subtilis* strain Bs96 and *P. fluorescens* strain T17-4 had the maximum effect on controlling the disease agent. By statistical analysis of this tests data, it became clear that the studied antagonists in current research can inhibit disease occurrence between 14.28 to 79.59% on unripe pear fruit. Also, host tissue inoculation with antagonists isolates before inoculation with fire blight agent is associated with the disease severity reduction. Many researchers have reported host tissue incubation with *E. herbicola*, an effective antagonist to control fire blight pathogen, or with yellow-color saprophits before attacking by *E. amylovora* led to decreasing disease severity (Thompson *et al*., 1976; Isenbeck and Schulz, 1985; Beer, 1981; Riggle and Klos, 1972). Also, in another study conducted by Ishimaru *et al*. (1988) on unripe pear test, they suggested that antagonist cells of *E. herbicola* C9-1 resulted in fire blight development in unripe pear fruit (Ishimaru *et al*., 1988). The results of Mirzai *et al*. (2015) research also showed that the isolates of *P. agglomerans* isolated from the apple gardens in Qazvin had the most inhibitory effect on bacterial growth by forming a 24 mm diameter barrier.

There were a concordance between results obtained from halo test and suppression in unripe pear fruit test. For example, *B. subtilis* strain BsVRU1, which showed the most suppression halo against pathogen, showed high biocontrol potential in unripe pear fruit test and 73.5% of the disease severity decreased. In addition, Andrews (1992) proved that many characters of the antagonist are effective in successful biocontrol. Of course the obtained results of initial selection could be acceptable only in that condition and any change in any variables such as temperature, medium, time and place of orchard and laboratory evaluation would change the results. However, many studies are required about microorganisms' traits, and yield difference in various microorganisms in

different conditions depends on environmental condition, host, and the existence of another biocontrol mechanisms. As natural condition is different from laboratory environment and microorganisms use all potentials to remain active and compete with the other ones, and for initial selection of the best antagonistic isolates against fire blight pathogen, the unripe pear fruit test was used since it provides more natural conditions compared to bacteria medium. Also, pear fruit was used because it is most sensitive to fire blight infection. Various mechanisms are effective in biocontrol potential antagonists such as bacteria, some of these mechanisms include the production of compounds like antibiotics, siderophores, hydrogen cyanide, ethylene and growth compounds (Phytohormones). Actually, these compounds count as metabolites that exist around bacteria as exteracellular extractions or volatile compounds (Kamilova *et al*., 2005). In order to make biocontrol applicable, understanding the exact mechanisms of the agent and a suitable formula will be of great help in disease control, so, the current study was conducted in assessment of antagonists' potential to control fire blight pathogen.

In this research, and similar to others for siderophore production, Vr87 isolate of *Enterobacter* sp. had the most impact after *B. subtilis* strain Bs96 and Vr5 isolate showed disease reduction by biofilm formation. The spectrophotometric method can only be used to determine the quantity of the pyoverdine type siderophore because different siderophores have different wavelengths. Since pyoverdine is the most important *Pseudomonas fluorescent* siderophore that has a higher iron content than other types of production and bond strengths (Cornelis and Matthijs, 2006), precise measurements can be used to determine the bacterial strain power in providing the iron needed for the plant and the study of the competitive control of the pathogens. The trait of fluorescence of the siderophore in a 400 nm wavelength allows a quantitative evaluation of the spectrophotometric method (Castaneda *et al*., 2005). Approximately, all cultivable microorganisms, except Lactobacillus, have the siderophore producing potential (Budzikiewicz, 1993). Many bacteria have the

capacity of induced resistance to disease in plants by producing siderophore (Hofte and Bakker, 2007). A number of researchers found that the production of pyoverdine in soil with biocontrol capacity of *fluorescent pseudomonas* is consistent (Becker and cook, 1988). However, there is still no information on the biocontrol action of pyoverdine at the aerial surfaces of plants, and only in a number of studies, the level of control of the isolates of the wild-type with the mutated isolates (incapable of producing pyoverdine) has been shown on the aerial surfaces of the plants (Lindow, 1988). The main role of siderophores is to provide iron for the cell (Leong, 1986) and it increases the amount of iron available in the cell and, as a result, increases the bacterial growth. Seidophobes can be considered as an important factor in increasing the survival capacity of biocontrol bacteria producing siderophore. Also, pyoverdine type siderophore is also effective in biofilm development. Therefore, The biofilm formation in Bacteria which live surrounding the philosopher is one of their most important strategies to tolerate conditions and factors such as temperature, humidity and light, also stress tolerance of UV exposure, nutritional deficiency, pH variables, antimicrobial compounds and things like that. As it is obvious, being unsustainable or reduced of survival of these bacteria in the environment are part of the difficulties in using beneficial microorganisms for biological control. In addition to increasing the resistance of bacteria to these stresses, biofilms enhance the ability of bacteria to transpose the horizontal gene and increase the production of secondary metabolites. Also, for a biocontrol agent, the biofilm is a useful place to initiate antagonistic interactions with the host (Charkowski *et al.*, 2008), while one of the problems with using useful microorganisms as biological control agents is the lack of establishment or reduction of their sustainability in the environment. So far, various research results have shown that biofilm plays an important role in colonizing bacteria (Stewart and William Costert, 2001). One of the benefits of biofilm formation for biocontrol agents is the continuous presence of bacteria at plant levels. Because of this presence, the biocontrol agent with the

biocontrol mechanisms available, including the production of antibiotics, and the high accumulation of its population in biofilms, has a great influence on the control of the pathogens and provides a stronger protection barrier against harmful agents. Biofilm is also considered as a defense mechanism in beneficial bacteria against other microorganisms (Molina *et al.*, 2003). Also, based on the production of secondary metabolites in high populations and complex systems of gene expression responsible for the production of various metabolites in bacteria in the biofilm phase, it can be seen that the biofilm plays a major role in the biocontrol of antagonists against pathogens.

As fire blight agent is an airborne one, direct impact of environmental condition on antagonist function occurs during use of biocontrol agents in orchards, and investigating isolate tolerance to drought stress and humidity deficiency is important. In the study of the effect of drought stress, although the increase of Polyethylene Glycol (PEG 6000) level in the NB culture medium caused a significant decrease in the growth rate of the selected antagonist agents, all antagonist agents were able to grow at a -30 bars level. The percent of growth decline in isolates Vr50 (5.17%) and Vr98 (6.10%) at -30 bars level was lower than the control without polyethylene glycol among other isolates and such results indicate that these two isolates are tolerant to drought stress conditions compared to others. The current study proved that Vr87 isolate of *Enterobacter* sp. had the most tolerance to drought stress in various osmotic potentials by light absorbance 1.29, 1.34, 1.38, 1.42, and 1.51 at -30, -20, -10 and -5 bar, respectively. Drought tolerance is one of the environmental factors that affect the products and leads to product reduction (Vmanchanda and Garg, 2008). In dry conditions, by decreasing the moisture, the population of the soil bacteria is reduced, but this population never reaches zero, so, some strains of the bacteria in the soil tolerate extremely dry conditions. These bacteria have benefited from water stored in micro-soil pores, and survived with minimal metabolic activity (Zahran, 2001). Some researchers have shown that some of the rhizobial bacteria (in saprophyte

conditions) could survive under drought stress or low water potential. A rhizobial strain isolated from dry soils can tolerate and grow in soil for about a month, while some commercial strains are unable to grow under these conditions (Zahran, 2001). One of the rapid signs of water stress on rhizobial bacteria is the bacterial morphological changes. Changes in rhizobia cells as a result of the effect of water stress, in the end, reduces the contamination and knotting of legumes (Worrall and Roughly, 1976).

In this research, after initial screening and selecting successful antagonistic isolates in biocontrol of fire blight agent by laboratory tests, selected isolates were used to investigate silver nanoparticle synthesis. With the advent of nanotechnology and given the antimicrobial properties of silver and the increase in this property on a nanoparticle scale, it can be used to combat various plants pathogens. Researchers have shown that biological systems can synthesize metallic particles with nanometer dimensions (Sadowski *et al.*, 2008). Synthesized microbial nanoparticles have different biological applications because of their environmental compatibility and compliance with the principles of green chemistry, non-toxic for human, and because of antibacterial and anti-fungal properties (Kalishwaralal *et al.*, 2008). Considering this property and considering the cost-effectiveness of nanoparticle synthesis by microorganisms, selective antagonistic isolates were evaluated for their synthesis ability. The results determined that *B. subtilis* strain BsVRU has the potential to release ions of silver and produce silver nanoparticles. Because the two-component system GacS/GacA regulates the production of bacterial metabolites (Dekkers, 1997), the regulation of the nitrate reductase enzyme may also be regulated by this system. It is likely that the system plays an important role in the creation of silver nanoparticles, which needs to be investigated. Kalishwaralal *et al.* (2008) reported that the mechanism of synthesis of silver nanoparticles in bacteria *Bacillus licheniformis* is likely to interfere with the bacteria reductase nitrate enzyme. This enzyme is stimulated by nitrate ions and converts silver ions into metallic silver. In fact, reductase nitrate acts as an electron transfer to a metal group. Studies have shown that NADH and the NADH-associated reductase nitrate released by this bacterium are important factors for converting Ag^+ to Ag and biosynthesis of metallic nanoparticles (Kalishwaralal *et al.*, 2008). Various researches have been conducted on the impact of the silver nanoparticles on controlling of and fighting with different types of microorganisms. Silver in large size is a metal with less reaction propertiese, but its capacity to eliminate microbes' increases up to 99% when it is converted to small size of nm (Slawson *et al.*, 1992). Many studies have been done on using these agents against plant pathogens. By the silver nanoparticles traits and the importance of fire blight pathogen on apple and pear, the impact of silver nanoparticles on growth of *E.amylovora* Ea.42 bacterium was tested. The obtained data identified that 60 ppm concentration of silver nanoparticles leads to 89.6% suppression and 10 and 30 ppm bacterial density can stop growth by 15.2 and 67.7%, respectively.

According to these data, *Enterobacter* sp. strain Vr87, *B. subtilis* strain BsVRU1 and *B. suubtilis* strain Bs96 are suitable in managing fire blight pathogen in apple and pear orchard. However, more investigation and pilot tests under orchards condition is needed to estimate the tolerance to biotic and non-biotic stress agents.

ACKNOWLEDGEMENTS

REFERENCES

1. Andrews, J. H. 1992. Biological Control in the Phyllosphere. *Annu. Rev. Phytopathol.*, **30**, 603-635.
2. Ausubel, F., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J., Smith, J. A. and Struhl, K. 1988. *Current Protocols in Molecular Biology.* Greene Publishing Associates and Wiley Interscience, New York, 427 PP.

3. Becker, J. and Cook, R. J. 1988. Role of Siderophores in the Suppression of Pythium Species and Production of the Increased-Growth Response of Wheat by Fluorescent Pseudomonas. *Phytopathology,* **78:** 778-782.

4. Beer, S. V. 1981. Towards Biological Control of Fire Blight. *Phytopathology,* **71:** 859.

5. Budzikiewicz, H. 1993. Secondary Metabolites from Fluorescent Pseudomonads. *FEMS Microbiol. Rev.,* **104(3):** 209–228.

6. Bugert, P. and Geider, K. 1995. Molecular Analysis of the Ams Operon Required for Exopolysaccharide Synthesis of *Erwinia amylovora. Mol. Microbiol.,* **15:** 917-933.

7. Castaneda, G. C., Munoz, T. J. J. and Videa, J. R. P. 2005. A Spectrophotometric Method to Determine the Siderophore Production by Strains of Fluorescent Pseudomonas in the Presence of Copper and Iron. *Microchem. J.,* **81(1):** 35-40.

8. Charkowski, A., Jahn, C., Apodaca, J. and Perna, N. 2008. A Horizontally Acquired Cellulose Synthase Operon in *Dickeya dadantii* Contributes to Biofilm Formation and Attachment to Plants. *Phytopathology,* **98**:S73.

9. Cornelis, P. and Matthijs, S. 2006. *Pseudomonas* Siderophores and Their Biological Significance. In: *"Microbial Siderophores"*, (Eds.): Varma, A. and Chincholkar, S. Springer-Verlag, Berlin, PP. 230-254.

10. Dekkers, L. C. 1997. Isolation and Characterization of Novel Rhizosphere Colonization Mutant of *Pseudomonas fluorescens* WCS365. PhD Thesis, Leiden University, Leiden, The Netherlands.

11. Giobbe, S., Marceddu, S., Scherm, B., Zara, G., Mazzarello, V. L. Budroni, M. and Migheli, Q. 2007. The Strange Case of a Biofilm-Forming Strain of Pichia Fermentans, which Controls Monilinia Brown Rot on Apple but Is Pathogenic on Peach Fruit. *FEMS Yeast Res.,* **7:** 1389-1398.

12. Goszczynska, T. Serfontein, J. and Serfontein, S. 2000. *Introduction to Practical Phytobacteriology. A Manual for Phytobacteriology.* ARC-Plant Protection Research Institute, SDC Switzerland. 83p. Disponible en linea: https://www.researchgate.net/publication/p ublication/237021880_Introduction_to_Pra ctical.

13. Gross, D. C., Lichens-Park, A. and Kole, C. 2014. *Genomics of Plant-Associated Bacteria.* Springer.

14. Heydorn, A., Nielsen, A. T., Hentzer, M., Sternberg, C., Givskov, M., Ersboll, B. K. and Molin, S. 2000. Quantification of Biofilm Structures by the Novel Computer Program COMSTAT. *Microbiology,* **146:** 2395-2407.

15. Höfte, M. and Bakker, P. A. 2007. Competition for Iron and Induced Systemic Resistance by Siderophores of Plant Growth Promoting Rhizobacteria. In: *"Microbial Siderophores"*, (Eds.): A. Varma, and S. P. Chincholkar, Berlin, Springer Verlag, PP. 121-133.

16. Hugh, R. and Leifson, E. 1953. The Taxonomic Significance of Fermentative versus Oxidative Metabolism of Carbohydrates by Various Gram-Negative Bacteria. *J. Bacteriol.,* **66(1):** 24-26.

17. Isenbeck, M. and Schulz, F. A. 1985. Biological Control of Fire Blight (*Erwinia amylovora* (Burr.) Winslow *et al.* on ornamentals. II. Investigation about the mode of action of the antagonistic bacteria. *J. Phytopathol*. **113**:324-333

18. Ishimaru, C. A., Klos, E. J. and Brubaker, R. R. 1988. Multiple Antibiotic Productions by *Erwinia herbicola. Phytopathology,* **78:** 746-750.

19. Johnson, K. B., Stockwell, V. O. and Vanneste, J. 2000. Biological Control of Fire Blight. *"Fire Blight: The Disease and Its Causative Agent, Erwinia amylovora"*, (Ed.): Vanneste, J. L. CAB International, Wallingford, UK, PPJ, **50:** 319-337.

20. Kalishwaralal, K., Ramkumar Pandian, S., Deepak, V., Mohd, B. and Sangiliyandi, G. 2008. Biosynthesis of Silver Nanocrystals by *Bacillus licheniformis. Colloids and Surfaces B: Biointerfaces*, **65:** 150-153.

21. Kamber, T., Lansdell, T. A., Stockwell, V. O., Ishimaru, C. A., Smits, T. H. and Duffy, B. 2012. Characterization of the Biosynthetic Operon for the Antibacterial Peptide Herbicolin in *Pantoea vagans* Biocontrol Strain C9-1 and Incidence in *Pantoea* Species. *AEM J.,* **78:** 4412-4419.

22. Kamilova, F., Validov, S., Azarova, T., Mulders, I. and Lugten Berg, B. 2005. Enrichment for Enhanced Competitive Plant Root Tip Colonizers Selects for a New Class of Biocontrol Bacteria. *Environ. Microbiol.,* **7:** 1809-1817.

23. Klement, Z., Farkas, G. L. and Lovrekovich L. 1964. Hypersensitive Reaction Induced by Phytopathogenic Bacteria in Tobacco Leaf. *Phytopathology,* **54:** 474.

24. Koczan, J. M., McGrath, M. J., Zhao, Y. and Sundin, G. W. 2009. Contribution of *Erwinia amylovora* Exopolysaccharides Amylovoran and Levan to Biofilm Formation: Implications in Pathogenicity. *Phytopathology,* **99:** 1237-1244.

25. Kovacs, N. 1956. Identification of *Pseudomonas pyocyanea* by the Oxidase Reaction. *Nature,* **178:** 703-703.

26. Lelliott, R., Billing, E. and Hayward, A. 1966. A Determinative Scheme for the Fluorescent Plant Pathogenic Pseudomonads. *J. Appl. Microbiol.,* **29:** 470-489.

27. Lelliott, R. A. and Stead, D. E. 1987. Methods for the Diagnosis of Bacterial diseases of Plants. *Blackwell Sci. Pub.,* **7:** 216

28. Leong, J. 1986. Siderophores: Their Biochemistry and Possible Role in the Biocontrol of Plant Pathogens. *Ann. Rev. Phytopathol.,* **24:** 187-209.

29. Lindow, S. 1988. Lack of Correlation of *in Vitro* Antibiosis with Antagonism of Ice Nucleation Active Bacteria on Leaf Surface by Non-Ice Nucleation Active Bacteria. *Phytopathology,* **78:** 444-450.

30. Manchanda, G., and Garg, N. 2008. Salinity and Its Effects on the Functional Biology of Legumes. *Acta Physiolog. Plantar.* **30:** 595-618.

31. Mayer, J. M. and Abdallah, M. A. 1978. The Fluorescent Pigment of *Pseudomonas fluorescens:* Biosynthesis, Purification, and Physic-Chemical Properties. *J. Gen. Microbiol.* **107:** 319-328.

32. Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J. and Wade, W. G. 1988. Design and Evaluation of Useful Bacterium Specific PCR Primers that Amplify Genes Coding for Bacterial 16S rRNA. *Appl. Environ. Microbiol.,* **64:** 795-799.

33. Michel, D. E. and Kaufmann, M. R. 1973. The Osmotic Potential of Polyethylene Glycol 6000. *Plant Physiol.,* **51:** 914-916.

34. Mirzai, M., Aminian, H. and Roustaee, A. 2015. The Study of Biological Control of Pear Fire Blight Caused by Erwinia amylovora by Some Antagonistic Bacteria. BSPPD, Spring, **1(1):** 39-47. (in Persian)

35. Molina, M. A., Ramos, J-L. and Espinosa-Urgel, M. 2003. Plant-Associated Biofilms. *Rev. Environ. Sci. Biotechnol.,* **2:** 99-108.

36. Petruta, C. C., Catalina, V., Matilda, C., Sorina, D., Manuela, C., Draganoiu, M., Valeriu, S. and Oncea, F. 2008. *In Vitro* Inhibition of *Erwinia amylovora* Romanian Isolates by New Antagonistic Bacterial Strains. *Rom. Biotechnol. Lett.,* **13(3):**1-10**.**

37. Rahnama, K. and Mazarei, M. 2002. The Status of Fire Blight on Pome Fruit in Iran. *Acta Hortic. (ISHS),* **590:** 99-102

38. Riggle, J. and Klos, E. 1972. Relationship of *Erwinia herbicola* to *Erwinia amylovora. Can. J. Bot.,* **50:** 1077-1083.

39. Sadowski, Z., Maliszewska, I. H., Grochowalska, B., Polowczyk, I. and Kozlecki, T. 2008. Synthesis of Silver Nanoparticles Using Microorganisms. *Materials Science-Poland,* **26:** 419-424.

40. Sakthivel, N. and Mew, T. 1991. Efficacy of Bacteriocinogenic Strains of *Xanthomonas oryzae* pv. *orezae* on the Incidence of Bacterial Blight Disease of Rice (*Oryza sativa* L.). *Can. J. Microbiol.,* **37**: 764-768

41. Sambrook, J., Fritsch, E. F. and Maniatis, T. 1989. *Molecular Cloning.* Cold Spring Harbor Laboratory Press, New York, **2:** 185.

42. Schaad, N., Braun-Kiewnick, A., Sands, D., Jones, J. and Chun, W. 2001. *Laboratory Guide for Identification of Plant Pathogenic Bacteria.* APS Press, Minnoesota, USA, 3: 398.

43. Singh, V. and Deverall, B. 1984. *Bacillus subtilis* as a Control Agent against Fungal Pathogens of Citrus Fruit. *Trans. Brit. Mycol. Soc.,* **83:** 487-490.

44. Slawson, R. M., Trevors, J. T. and Lee, H. 1992. Silver Accumulation and Resistance in *Pseudomonas stuuzeri. Arch. Microbiol.,* **158:** 398-404.

45. Slawson, R. M., Van Dyke, M. I., Lee, H. and Trevors, J. T. 1992. Germanium and Silver Resistance, Accumulation, and Toxicity in Microorganisms. *Plasmid,* **27:** 72-79.

46. Stenstrom, I. M., Zakaria, A. Ternstrem, A. and Molin, G. 1990. Numerical Taxonomy of *Fluorescent pseuodomonas* Associated with Tomato Roots. *Antonie Van Leeuwenhoek,* **57:** 223-236.

47. Stewart, P. S. and William Costerton, J. 2001. Antibiotic Resistance of Bacteria in Biofilm. *The Lancet,* **358:** 135-138.

48. Stockwell, V., Johnson, K., Sugar, D. and Loper, J. 2002. Antibiosis Contributes to Biological Control of Fire Blight by *Pantoea agglomerans* Strain Eh252 in Orchards. *Phytopathology, 92:* 1202-1209.

49. Suslow, T., Schroth, M. and Isaka, M. 1982. Application of a Rapid Method for Gram Differentiation of Plant Pathogenic and Saprophytic Bacteria without Staining. *Phytopathology,* **72**:917-918.

50. Thompson, S. V., Schroth, M. N., Moller, W. J., and Reil, W. O. 1976. Efficacy of Bacteriocides and Saprophytic Bacteria in Reducing Colonization and Infection of Pear Flowers by *Erwinia amylovora. Phytopathology,* **66:** 1457-1459.

51. Thornley, M. J. 1960. The Differentiation of Pseudomonas from Other Gram Negative Bacteria on the Basis of Arginine Metabolism. *J. Appl. Bacteriol.,* **13:** 37-52.

52. Van der Zwet T. 2002. Present Worldwide Distribution of Fire Blight. *Acta Hortic. (ISHS),* **590:** 33-34.

53. Vmanchanda, I. and Garg, N. 2008. Salinity and Its Effects on the Functional Biology of Legumes. *Acta. Physiol. Plant..,* **30(5)**:595-618.

54. Worrall, V. S. and Roughly, R. J. 1976. The Effects of Moisture Stress on Infection of *Trifolium subteerraneum* L. by *Rhizobium trifoli* Dang. *J. Exp. Bot.,* **27:** 1233-1241.

55. Zahran, H. H. 2001. Condition for Successful Rhizobium-Legume Symbiosis Saline Environment, *Biol. Fertil. Soil.,* **12:** 73-80.

تأثیر برخی باکتری های پروبیوتیک بر *Erwinia amylovora* عامل ایجاد کننده بیماری آتشک

م. پورجعفری، ر. صابری ریسه، پ. خدایگان، ا. حسینی‌پور، و م. مرادی

چکیده

بیماری آتشک (Fire blight) جزو قدیمی‌ترین بیماری‌های گیاهی و ازجمله بیماری‌های مهم درختان میوه دانه‌دار (گیاهان خانواده Rosaceae) خصوصاً سیب و گلابی در جهان محسوب می‌شود که به‌وسیله باکتری *Erwinia amylovora* ایجاد می‌شود. امروزه با توجه به مشخص بودن زیان‌های سموم شیمیایی برای انسان و محیط‌زیست، اهمیت کاربردی کنترل زیستی برای مبارزه با بیماری‌های گیاهی آشکارشده است. علاوه بر ملاحظات زیست‌محیطی، ازآنجایی‌که روش‌های مطمئنی برای مبارزه با بیماری آتشک سیب و گلابی، در دست نیست، کنترل بیولوژیک این بیماری، از اهمیت ویژه‌ای برخورداراست. به‌منظور بررسی پتانسیل برخی باکتری‌های پروبیوتیک به‌عنوان عوامل کنترل بیولوژیک در مقابل پاتوژن آتشک، در سال ۱۳۹۳ نمونه‌هایی از شاخ و برگ سالم درختان سیب، گلابی و به، از مناطق مختلف استان کرمان جمع‌آوری شد و فعالیت بیوکنترلی عوامل آنتاگونیست، در شرایط آزمایشگاه مورد ارزیابی قرار گرفت. نتایج ارزیابی آزمایشگاهی نشان داد که برخی از جدایه های باکتریایی، توانستند سبب کاهش ۱۴.۲۸ تا ۷۹.۵۹ درصدی علائم بیماری گردند. ارزیابی آزمایشگاهی ازجمله بررسی کاهش شدت بیماری در میوه گلابی نارس، هاله ممانعت از رشد، تولید سیدروفور بازدارنده، توان تشکیل بیوفیلم، توان تحمل تنش خشکی و توان سنتز نانو ذرات نقره نشان داد که این جدایه ها پتانسیل کنترل این بیماری رادارند. از بین ۹ جدایه برتر آنتاگونیستی در آزمون گلابی نارس در کنترل پاتوژن آتشک، که از باغات سیب و گلابی جداسازی شده بودند، جدایه

Vr87 به نمایندگی انتخاب و جهت شناسایی، بخشی از ناحیه 16S rDNA آن با به‌کارگیری آغازگر اختصاصی تکثیر و تعیین ترادف شد. با مقایسه نتایج در پایگاه اینترنتی NCBI، جدایه منتخب به‌عنوان Enterobacter sp. شناسایی شد. در بین تمام جدایه های منتخب به‌عنوان عوامل موفق در کنترل پاتوژن آتشک اعم از جدایه های جداسازی شده و جدایه های موجود در کلکسیون دانشگاه ولی‌عصر(عج)، جدایه BsVRU1 استرینی از جنس _Bacillus subtilis_ موجود در کلکسیون بیماری شناسی دانشگاه ولی‌عصر (عج)، با ۷۳.۵ درصد قدرت بازدارندگی بهتری نسبت به سایر جدایه های آنتاگونیست در مقابل پاتوژن بیماری آتشک دارا بود.