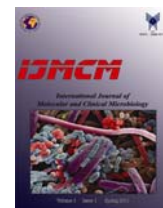




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### Antagonistic potential of rhizospheric and endophytic bacteria against Fire blight, caused by *Erwinia amylovora*

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#### ABSTRACT

Fire blight, caused by *Erwinia amylovora*, is one of the most important diseases of fruit trees worldwide. The aim of this study was to isolate and identify rhizospheric and endophytic bacteria with antagonistic activity against *Erwinia amylovora* in apple and pear orchards around Gorgan, Golestan province. Root, leaf and rhizospheric soil samples were cultured on nutrient agar medium and after incubation morphological features of the appeared colonies were examined. The antagonistic activity of the isolates was determined by well diffusion agar. Chloroform test was used to evaluate the production of antimicrobial agent by antagonist isolates and catalase and protease sensitivity tests were used to determine its nature. The thermal stability of the antimicrobial agent and the effect of pH on its inhibitory activity were also evaluated. Isolates with more antagonistic activity were identified based on 16S rRNA sequencing. Fourteen isolates produced antimicrobial substance with antagonistic activity, which had a nature other than hydrogen peroxide. The antimicrobial agents from 8 isolates were proteinaceous in nature. The inhibitory activity of cell-free supernatant of these isolates was inactivated at 100 °C and had the best effect at neutral pH. The isolates identified by molecular method had a more than 90% similarity to *Bacillus subtilis* strain B-12, *Bacillus subtilis* strain YL-3, *Paenibacillus polymyxa* strain DST34, *Pantua aglomrants* strain ACBP1 strain. In the present study, bacteria with antagonistic activity against *E. amylovora* were isolated from rhizosphere and endophyte, but to better judge their performance, more tests are needed in different conditions.

#### 1. Introduction

Fire blight, caused by *Erwinia amylovora*, is one of the most important diseases of fruit trees worldwide. In this disease, the infected plant first rapidly wilts, then becomes necrotic, and finally turns into blackish-brown (Zhao et al., 2005). This bacterium infects most ornamental and economic cultivars belonging to the rosacea family, especially important trees such as apple and pear trees (Vrancken et al., 2013). Fire blight usually causes severe

economic damage to the commercial production of these trees, so that in the United States, the damage is estimated at about \$ 100 million annually due to reduced production and disease management costs. In Iran, this disease was first reported in 1989 in pear trees of Karaj orchards and now in many areas of apple and pear orchards. According to the latest official report, the amount of pollution in apple and pear

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orchards in Tehran province is estimated at about 20,000 hectares (Gerami et al., 2013).

Fire blight is difficult to control because it spreads rapidly in the plant and there are no effective ways to control it (Vrancken et al. 2013). To control, the infected parts of the tree and sometimes the whole tree is cut down. To prevent disease, trees are sprayed with copper compounds, but this does not cure infected plants. Copper can also cause side effects in the form of rust on leaves and fruits, especially for apples. In addition, the European Union has restricted the use of copper in fruit crops due to its accumulation in the soil and adverse effects on living organisms. In some countries, such as the United States, New Zealand, Canada, and Mexico, the use of antibiotics is recommended (Mikiciński et al., 2020). Streptomycin or oxytetracycline antibiotics have been used to control the disease during the flowering period, but resistance of *E. amylovora* to these antibiotics has been observed in many areas (Pusey, 2002, Piqué et al., 2015).

Today, the World Health Organization and the European Union have banned the use of antibiotics in the treatment of plant diseases, and on the other hand, concerns about the dangers of using chemical pesticides for humans and the environment are increasing (Mirzaei et al., 2012). Therefore, due to these limitations, control of this disease using alternative strategies is inevitable. In recent years, interest in biological control of plant diseases as one of the alternative methods has increased. Biocontrol agents are usually fungi, bacteria, or viruses or a mixture of them that act specifically and affect only the target species (O'Brien, 2017). One of these biological agents is *Pseudomonas fluorescens*, which can inhibit plant diseases by producing a number of secondary metabolites, including antibiotics, siderophores and hydrogen cyanide (Ganeshan and Kumar, 2005), so that in a study the use of *P. fluorescens* and *Pantua agglomerans* C9-1 or their antagonist derivatives reduced tree blossom blight from 43% to 83% (Sundin et al., 2008). Inhibition of *E. amylovora* on tree blossoms using antagonistic bacteria has been considered as an alternative method in controlling fire blight (Gerami et al. 2013). In recent years in some parts of the country, this disease has caused a lot of damage to fruit trees and few studies have been done on its biological control (Baba-Ahri et

al., 2007). Therefore, the purpose of this study was to isolate and identify of rhizospheric and endophytic bacteria with antagonistic activity against *E. amylovora* in apple and pear orchards around Gorgan city located in Golestan province.

## 2. Materials and Methods

### 2.1. Isolation of endophytic bacteria

In this study, the roots and leaves of apple and pear trees in the orchards of Marzankola and Fazelabad villages were sampled. The samples were immediately transferred to the laboratory. First, the samples were washed several times with water to remove soil, excess particles and surface germs. Then they were placed in 70% ethanol for 1 min, in 2.5% sodium hypochlorite for 20 min and again in 70% alcohol for 30 sec and finally washed three times with sterile distilled water. One ml of the last wash fluid was cultured in nutrient agar medium as a control. The disinfected roots and leaves under sterile conditions were cut into 1 to 2 mm pieces in 200  $\mu$ l of sterile distilled water and ground in a sterile mortar. After 30 min, a loop of suspension was cultured on nutrient agar medium and heated at 26 ° C for 3 to 5 days. The colonies were then isolated and purified based on morphology (size, color and shape) (Liaqat and Eltem, 2016, Kurd et al., 2010). Initial identification of isolates was performed by gram staining and spore production ability.

### 2.2. Isolation of rhizospheric bacteria

Ten soil samples were randomly sampled from the rhizosphere of trees. Ten g of the sieved soil sample were poured into an Erlenmeyer flask containing 90 ml of sterile saline and shaken gently for 5 min to obtain a uniform suspension of soil. The resulting suspension was diluted using sterile saline to obtain a  $10^{-6}$  dilution. From this dilution, 0.1 ml was poured on the surface of the nutrient agar medium and spread with a sterile spreader. After drying the culture surface, the plates were incubated at 37°C for 48 h. The grown colonies were then isolated and purified based on morphology (size, color and shape) (Akbarpour and Behboudi 1398). Gram staining and spore production ability were used for initial identification of the isolates.

### 2.3. *In vitro* evaluation of antagonistic activity

The antagonistic activity of the isolates was investigated by agar well diffusion method. The isolates were transferred individually to 50 ml of Luria-Bertani broth in a 250 ml Erlenmeyer flask and incubated on a shaker at 200 rpm for 4 days at room temperature. Twenty ml of Luria-Bertani agar was poured into sterile plates with a diameter of 90 mm. One ml of bacterial suspension of *E. amylovora* (108 CFU / ml) was mixed with 3 ml of Luria-Bertani agar (0.6% agar) at 45°C and spread rapidly on the plates containing Luria-Bertani agar. Wells with a diameter of 6 mm were then punched in the agar. To obtain a cell-free supernatant, the pre-culture isolates were centrifuged for 30 minutes at 15,000 rpm to remove cell debris, then 100 µl of each sample was sterilized with a 0.45 µm filter and poured into wells. The plates were incubated at 26°C for 3 to 5 days and the growth inhibition zone around the wells was measured. Two replications and two separate experiments were performed for each isolate (Kird et al. 2010).

### 2.4. Antimicrobial production test

Chloroform test was used to evaluate the production of antimicrobial agent by antagonist isolates. To perform this test, the isolates were streaked in nutrient agar medium and after 72 h of incubating at 28°C, the bacteria were washed from the plate surface with sterile distilled water. A piece of chloroform-impregnated cotton was then placed on the plate lid and placed upside down. After 20 min when the chloroform vapor was completely removed from the plate, 200 µl of *E. amylovora* suspension with a concentration of 10<sup>3</sup> CFU/ml was uniformly spread on the surface. The plates were incubated at 28°C for 48 h. Then, the results were analyzed by counting *E. amylovora* colonies (Mirzaei et al., 2012).

### 2.5. Determining the nature of the antimicrobial agent

Catalase susceptibility test was used to determine the antimicrobial nature of antagonist isolates. To perform this test, 1 ml of cell-free supernatant was incubated for 3 h at 37°C in the presence of catalase (1 mg / ml, Sigma-Aldrich, Germany). Cell-free supernatant was considered

as the control. The presence of an antibacterial compound other than hydrogen peroxide was confirmed in the existence of a growth inhibition zone around the catalase wells. Susceptibility of antibacterial compounds to proteolytic enzymes was determined after incubating the cell-free supernatant for 1 h at 37°C with proteinase K and alkaline protease (both enzymes 1mg/ml, Sigma-Aldrich, Germany). After incubating, these enzymes were inactivated for 3 min at 100°C. Then the presence or absence of a growth inhibition zone around the wells was examined. In the absence of a growth inhibition zone, the antimicrobial substance was confirmed to be protein

### 2.6. Effect of temperature and pH on antibacterial activity

Thermal stability of antibacterial activity was determined by incubating 500µl of cell-free supernatant at 40, 60, 80 and 100°C for 30 min. The activity of antibacterial compounds at different pH values was determined after placing the supernatant for 1 day at 4°C in phosphate buffers with pH 3 to 9. After incubating to a certain pH before assay the medium was neutralized (Kird et al., 2010).

### 2.7. Identification of antagonist isolates based on 16S rRNA gene sequence

To extract DNA, a colony from the antagonist isolate was inoculated in Luria-Bertani and incubated at 37°C for 24 h. Then, using a DNA extraction kit (Kiagen, Germany), 1 ml of this culture was used for extraction according to the manufacturer's instructions. The quality and quantity of DNA extracted was determined on 1% agarose gel. 16S rRNA amplification was performed by PCR reaction using a pair of universal primers whose sequences are shown in Table 1. For PCR, 1 µl of each primer (20 pmol), 5 µl of DNA and 19 µl of sterile distilled water were added to Mastermix 2X (Sinagen, Iran). Amplification was performed in 30 cycles including denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and elongation at 72°C for 30 seconds. Initial denaturation and final elongation were performed at 94°C for 3 min and 72°C for 10 minutes, respectively. The PCR product were detected by electrophoresis on a

1% agarose gel and purified using gel extraction kit (Qiagen, Germany). Amplified PCR products were purified using a Gel Purification Kit (Qiagen, Germany) and were submitted to South Korea Macrogen Company for sequencing.

The obtained sequences were blasted against existing sequences in GeneBank (Ahani Azari et al., 2019).

**Table 1.** Primers used in this study (Weisberg et al., 1991)

Primer	Sequences (5→3)	product PCR
16S-F	5'-AGAGTTTGATCCTGGCTCAG 3'	1500bp
16S-R	5'-ACGGCTACCTTGTTACGACTT3'	

### 3. Results

A total of 37 bacterial strains were isolated from the roots (13), leaves (7) and rhizosphere (17), respectively. Among the isolates, 20 isolates were gram positive and 17 isolates were gram negative. Based on the results of antagonistic activity of the isolates, 14 isolates showed antagonistic activity against *E. amylovora*, of which 11 isolates were spore-forming gram-positive bacilli and 3 isolates were gram-negative bacilli. Among the isolates, R7 and R5 isolates showed the highest and lowest inhibitory activity against *E. amylovora* (Table 2), respectively, which was consistent with the results obtained from the chloroform test, i.e., compared to the control, R7 isolate with a growth inhibition zone of 20 mm in diameter produced the highest and R5 isolate with a growth inhibition zone of 6 mm in diameter produced the lowest amount of antimicrobial agent (Figure 1).

In the catalase test, it was found that the antimicrobial substance produced by the isolates is of a nature other than hydrogen peroxide. The antimicrobial produced by 8 of these isolates was inactivated after treatment with proteases, indicating their protein nature, while the antimicrobial produced by other isolates was not inactivated by these treatments and probably had no protein nature. The inhibitory activity of the isolate-free supernatant was eliminated at 100°C while other heat treatments were ineffective. Regarding the effect of pH changes on inhibitory

activity, it was found that cell-free supernatant of all isolates had the best effect at neutral pH.

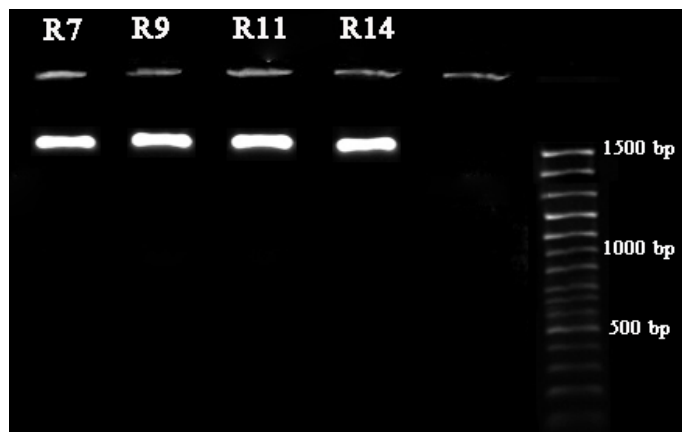
The four isolates R7, R9, R11 and R14, which had the highest inhibitory activity against *E. amylovora*, were identified based on the 16S rRNA gene sequence. The PCR result has been shown in Figure 2. After sequencing of 16S rRNA gene, it was found that these isolates were more than 90% similar to *Bacillus subtilis* strain B-12, *Bacillus subtilis* strain YL-3, *Paenibacillus polymyxa* strain DST34, *Pantoea agglomerans* strain ACBP1, respectively (Table 3).



**Figure 1.** Growth inhibition zone produced by antagonist isolates

**Table 2.** Characteristics and mean diameter of antagonist isolate growth inhibition zone

Mean diameter of growth inhibition zone (mm)	Sampling location	Sample type	Spore formation	Gram staining	Isolates
12	Fazelabad	Soil	+	+	R1
8	Marzankola	Soil	+	+	R2
11	Fazelabad	Soil	+	+	R3
10	Marzankola	Soil	+	+	R4
6	Fazelabad	Soil	+	+	R5
12	Fazelabad	Soil	+	+	R6
20	Marzankola	Soil	+	+	R7
11	Fazelabad	Soil	+	+	R8
18	Marzankola	Soil	+	+	R9
13	Fazelabad	Soil	+	+	R10
15	Fazelabad	Apple root	+	+	R11
9	Marzankola	Apple leaves	-	-	R12
7	Fazelabad	Pear leaf	-	-	R13
16	Marzankola	Pear leaf	-	-	R14

**Figure 2.** Lane 1: DNA ladder 1kb as a size standard, Lanes 2-5: PCR result on the genomic DNAs of R7, R9, R11 and R14 strains

**Table 3.** Identification of isolates based on 16S rRNA gene sequencing

Percentage of similarity	Accession number	Strains	Isolates
97	HQ009797.1	<i>Bacillus subtilis</i> strain B-12	R7
96	GQ262727.1	<i>Bacillus subtilis</i> strain YL-3	R9
96	MT163461.1	<i>Paenibacillus polymyxa</i> strain DST34	R11
95	KY357287.1	<i>Pantoea agglomerans</i> strain ACBP1	R14

#### 4. Discussion

Similar studies have been conducted in the country and different countries on biocontrol of fire blight, including the Pusey study, which demonstrated the efficacy of *P. agglomerans* strain E325 in controlling the *E. amylovora* and reducing the prevalence of apple blight (Pusey 2002). A study in France also reported the production of antibiotics by the epiphytic bacterium *Erwinia herbicola* strain Eh252 and its role in the biological control of *E. amylovora* (Vanneste et al., 1992). Gerami et al. also studied the role of bacterial antagonists in inhibiting fire blight in laboratory and garden conditions, with the highest and lowest inhibitory effects in the garden related to *P. agglomerans* strain Abp2 and *Serratia marcescens* strain Kgh1, respectively (Gerami et al., 2013). In a study in Poland, Mikiciński et al. studied the biocontrol of eight bacteria isolated from the apple philosopher against *E. amylovora*, among which three isolates of *Pseudomonas chlororaphis* subsp. *aureofaciens*, *Pseudomonas congelans* and *Pseudomonas protegens* were able to inhibit the growth of pathogen on the LB or R2A medium (Mikiciński et al. 2017).

A study in Belgium also showed the protective role of *Serratia plymiticum* against fire blight of blossoms and fruits of pear trees (Schoofs et al., 2002). Spanish researchers also reported that *Pseudomonas fluorescence* strain EPS62e were 90% effective in inhibiting *E. amylovora*, and

*Lactobacillus plantarum* isolates were able to protect 28 to 68% of the leaves against this pathogen (Roselló et al., 2013). In a study, Eb660 and Eb661 strains of *Erwinia billingiae* were shown to be highly effective in protecting pear fruit and apple blossoms against fire blight (Jakovljevic et al., 2006). In a recent study in Morocco, Bahadou et al. reported new antagonists for the control of *E. amylovora*, belonging to the genera *Alcaligenes*, *Serratia*, and *Brevibacterium* (Bahadou et al., 2018). In Poland, Mikiciński et al. also isolated the *Pseudomonas graminis* from apple philosopher, which could protect the plant from fire blight caused by *E. amylovora* (Mikiciński et al., 2016).

In the present study, bacteria with antagonistic activity against *E. amylovora* were isolated from rhizosphere and endophyte, but more experiments in different environmental conditions are needed to better judge their performance. However, the results of this study, consistent with the results of other researchers, showed the rhizospheric and endophytic bacteria especially *Bacillus* spp. have a great potential to be used as biocontrol for the plant diseases.

One of the limitations of the present study was that the inhibitory activity of antagonist isolates in garden conditions was not investigated due to the lack of cooperation of gardeners at the sampling site. In future studies, to introduce effective biocontrol agents in plant diseases, it is recommended to study the antagonistic

activity of isolates in laboratory and garden under different environmental conditions.

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