عوامل شایع بیماری شانکر باکتریای زردآلو از استان همدان

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چکیده

مقدمه: استان همدان به‌دلیل شرایط آب‌وهوایی مناسب یکی از مهم‌ترین مناطق برای کاشت درختان هسته‌دار مانند زردآلو (*Prunus armeniaca*) است. بیماری شانکر درختان هسته‌دار ناشی از *Pseudomonas syringae pv. syringae* یکی از جدی‌ترین بیماری‌ها در این استان، ایران و سراسر جهان است. مطالعه به‌منظور شناسایی عامل بیماری انجام شد.

مواد و روش‌ها: نمونه‌های زردآلوی بیمار در اوایل بهار و اواخر پاییز سال ۱۳۹۷ جمع‌آوری شدند. از نمونه‌های جمع‌آوری‌شده، ۱۳۰ استرین باکتریایی جداسازی شدند که ۶۵ استرین براساس رنگ و نوع پرگنه‌ها برای مطالعات بیشتر انتخاب شدند. از روش SDS-PAGE برای گروه‌بندی استرین‌های نماینده استفاده شد. بیماری‌زایی استرین‌ها در نهال‌های زردآلو در شرایط گلخانه بررسی شد. استرین‌های مطالعه‌شده براساس ویژگی‌های فنوتیپی، با استفاده از روش‌های استاندارد باکتری‌شناسی و بررسی توالی ژن 16S rRNA شناسایی شدند.

نتایج: ۳۰ استرین، حساسیت زیادی روی برگ‌های شمعدانی نشان دادند. با توجه به الگوی پروتئین‌های محلول سلولی روی ژل پلی‌اکریل‌آمید، استرین‌ها در ۷ گروه قرار گرفتند. نتیجهٔ بلاست توالی ژن 16S rRNA از این استرین، تشابه ۹۸/۷ درصد با *P. syringae* NCPPB 281 را نشان داد. استرین دیگر (استرین FB46) به‌دلیل تشابه ۹۵/۳۳ درصد با *Pantoea agglomerans* RSG19 توسط بلاست توالی ژن 16S rRNA، به‌عنوان *P. agglomerans* شناخته شد.

بحث و نتیجه‌گیری: نتایج به‌دست‌آمده با یافته‌های واصبی و همکاران مطابقت دارد. آنها *P. syringae* را به‌عنوان عامل بیماری شانکر زردآلو در استان آذربایجان شرقی جدا کردند. همچنین، *P. agglomerans* به‌عنوان یکی از عوامل مرتبط با میوه‌های هسته‌دار، میوه‌های دانه‌دار و درختان گردو در استان البرز گزارش شده است. این نخستین گزارش از وجود *Pseudomonas syringae* به‌عنوان عامل بیماری شانکر زردآلو در استان همدان است و باکتری *Pantoea agglomerans* برای نخستین‌بار در جهان به‌عنوان عامل ایجادکنندهٔ علائم شانکر زردآلو معرفی شده است.

واژه‌های کلیدی: بیماری شانکر زردآلو، *Pseudomonas syringae*، *Pantoea agglomerans*، 16S rRNA

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Prevalent causal agents of apricot bacterial canker disease from Hamedan province

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Abstract

Introduction: Hamedan province is one of the most important regions for cultivation of stone fruit trees, such as apricots (*Prunus armeniaca*) due to its suitable climatic conditions. Bacterial canker disease of stone fruit trees caused by *Pseudomonas syringae* pv. *syringae*, is one of the most serious diseases in this province, Iran and all over the world. The study was performed to identify the causative agent of the disease.

Materials and Methods: Samples of diseased apricots were collected in early spring and fall autumn of 2018. From the collected samples a total of 130 bacterial strains were isolated of which 65 representative's strains were selected based on the color and type of colonies on the culture medium for further study. SDS-PAGE technique was used to group the representative's strains. Pathogenicity of the representative's strains were performed on apricot seedlings under greenhouse condition. The studied strains were identified based on their phenotypic features, using standard bacteriological method and followed by sequencing of the 16S rRNA encoding gene.

Results: 30 strains showed hypersensitivity reaction on geranium leaves. According to the pattern of cellular soluble proteins on polyacrylamide gel (SDS-PAGE), the strains were divided into seven groups. BLAST of partial sequence of 16S rRNA encoding gene from this strain showed 98/7% similarity to *P. syringae* NCPPB 281. Another strain (FB46 strain) was identified as *P. agglomerans* due to 95.33% similarity with *Pantoea agglomerans* RSG19 by BLAST of 16S rRNA encoding gene sequence.

Discussion and Conclusion: The results obtained are in agreement with of Vasebi *et al*. isolated *P. syringae* as a causative agent of apricot canker in East Azerbaijan province. *P. agglomerans* has also been reported as one of the factors associated with stone fruits, pome fruits and walnut trees in Alborz province. This is the first report for the presence of *Pseudomonas syringae* as a causative agent of apricot canker disease in Hamedan province. The bacterium *Pantoea agglomerans* is reported from the first time in the world as the causative agent which induce apricot canker symptoms.

Key words: Apricot canker disease, *Pseudomonas syringae*, *Pantoea agglomerans*, 16S rRNA

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Introduction

Stone fruit trees are often endemic to temperate regions belonging to the family of *Rosaceae*, a subfamily of *Prunoideae* and *Prunus* genus. This large genus includes plum, peach, chile, greengages, cherry, apricot, almond, and many species, some are used as the base or the ornamental plant (1). Apricot or by scientific name *Prunus armeniaca* L., belongs to the family *Rosaceae*, subfamily *Prunoideae*, genus *Prunus*. *Prunus* has many species that are economically important for the production of fruit, nuts, wood, and as an ornamental plant for the green space (2).

According to the latest FAO statistics in 2014, Iran is the second-largest producer of apricots in the world after Uzbekistan with 432,000 tons. According to FAO statistics in 2017, Iran has 11547 hectares of apricot cultivated area with a production rate of 239712 tons and a yield equivalent to 207597 kg/ha, Hamedan province accounts for 3.9% of this amount. According to the latest FAO statistics in 2020, Turkey is the first apricot producer in the world with 730 thousand tons of apricots, followed by Uzbekistan and Iran with 662 and 306 thousand tons, respectively. (3).

Diseases such as stone fruit trees rust (*Tranzchelia pruni-spinisa* D.), the complexity of peach leaf and plum deformity (*Taphrina deformans* Tul., *T. pruni* Tul.), stone fruit shot hole spot (*Wilsonomyces carpophilus* M.B. Ellis), peach powdery mildew (*Sphaerotheca pannosa* de Bary), Mummification (*Monilinia fructicola* Honey, *M. fructigena* Honey), stone fruit trees *cytospora* canker (*Cytospra leucostoma* Höhn), necrotic ring spot (*Prunus necrotic ring spot* G.P. Martelli), stone fruit trees leaf spot (*Xanthomonas arboricola* pv *pear* Vauterin *et al.*), and stone fruit bacterial canker (*Pseudomonas syringae* pv *syringae* Van Hall), cause economic losses to the stone fruit trees each year (4).

Bacterial species belonging to the *Pseudomonas* genus are among the most important plant pathogens. These bacteria are distributed worldwide and reduce crop quantity and quality. A few pathovars of *P. syringae* such as *P. s.* pv. *syringae* (*Pss*) cause canker disease on stone fruits. Hamedan province is one of the important regions for the cultivation of stone fruit trees, such as apricots (*Prunus armeniaca*), due to its suitable climatic conditions (5). This disease causes a 10 to 20% reduction in yield and sometimes this damage reaches up to 80%, and even 10 to 75 percent of trees are lost in young gardens. Symptoms include cankers with leachate leaking from the branches, twigs, and trunks of the tree. These symptoms also occur in pruning wounds. Infection begins and the disease progresses in late winter and early spring. Leachate or gum is produced in most cankers and seeps out by contaminating the site (5). Cankers that do not produce gum are softer, wetter, and sunken and may have a sour smell. Due to the spread of canker, the upper part of the plant is cut off from the lower parts, and the leaves become twisted and wilted, and then the branch or all the upper parts of the tree may dry out (5).

Pseudomonas syringae is a gram-negative bacterium, aerobic, the subtype gamma-proteobacteria, rod-shaped, with one or more polar flagella and with few exceptions producing fluorescent pigments. The main hosts of this pathogenic bacterium are cherry, peach, and apricot trees, as well as almond, pear, citrus, maple, alder, hazelnut, magnolia, lilac, poplar, lily, rose, a number of vegetables, and cereal (6).

Materials and Methods

Sampling and Isolation: In this study, samples of apricot diseased trees in Hamedan province were collected in early spring and late autumn of 2018. The collected samples included young and infected middle branches of stone fruits trees, which were kept in the refrigerator until culture. To isolate the causal bacteria agents of the disease, samples were washed with tap water followed by sterilized distilled water. Pieces of plant tissues from the border of healthy and infected tissues were separated, chopped in Petri dishes, and put in a few drops of sterilized distilled water for 30 minutes. A loopful of bacterial suspension was a stroke on nutrient agar medium (NA), kept for two to three days at 26-27 °C. The grown single colony was then isolated from the medium and purified on a nutrient agar medium for daily use. For longer-term storage, from freshly cultured bacteria (24 hours), a concentrated suspension was prepared in microtubes containing sterile distilled water and stored in a refrigerator at 4 °C (6).

Hypersensitivity reaction (HR): Most plant pathogenic bacteria have the ability to cause hypersensitivity to tobacco or geranium. In this experiment, a relatively opaque suspension of bacteria with an approximate concentration of 10^8–10^{11} CFU / ml was prepared and injected by a sterilized insulin syringe between two-leaf epiderma of geranium (6).

Extraction of total soluble cell protein from samples: Bacterial strains were cultured on a NA medium for 48 hours, their cell mass was collected by a sterile loop from the surface of the medium and poured into 1.5 ml microtubes. For every loop of bacteria (3 ml^3), 1000 µl of protein extraction buffer was added, shake vigorously with a vortex device three times per minute. The microtubes were immersed in boiling water for five minutes and immediately were placed in an ice-water mixture for two minutes. The microtubes were centrifuged at 10,000 rpm for 10 minutes, the upper phase of protein suspension was gently removed and kept in a new microtube and kept in a freezer at -20 °C until using. For comparison of cellular proteins of strains isolated from apricot trees, protein electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed in the discontinuous Laemmli system (6, 7).

Pathogenicity test: Since *P. s.* pv. *syringae* (*Pss*) survival on the plant surface is possible, therefore, the presence of the bacteria with the plant tissues does not prove its pathogenicity and in this case, pathogenicity tests should be performed (8). The pathogenicity test was performed according to Tomidis et al. (2005), by injection of bacterial suspension (10^3 -10^5 CFU/ml) prepared from freshly grown culture into young plant tissues. The injected plants were kept in greenhouse conditions ($25^{\circ C}$ ± 3) and the emergence of canker and longitudinal sunken wounds after 10 to 15 days was considered a positive sign of the pathogenicity test. Three replicates were applied to each strain. They were examined for approximately one month for complete assurance (9).

Characterization of the phenotypic properties of the bacterial strains: Identification of purified pathogenic strains was performed according to standard bacteriological methods (6). The gram reaction of bacterial strains was determined by the solubility test in potassium hydroxide 3% (6).

Polymerase chain reaction (PCR): In order to extract the DNA from bacterial cells, they were cultured on NA medium for three days, a loop of bacteria was poured into the microtube and 1000 µl of distilled water or TE buffer was added to them and boiled three times in warm water bath for 1.5 minutes and immediately cooled. The microtubes were centrifuged at

13,000 rpm for 10 minutes and a microliter of the upper phase was used for polymerase chain reaction (10).

DNA electrophoresis in agarose gel: This method was used to estimate the quantity and quality of extracted DNA and its non-fracture. For this purpose, four microliters of DNA were extracted for each sample, mixed with two microliters of buffer or loading dye, and poured into a 0.8% agarose gel well. The gel was electrophoresed for 1.5 hours at a constant voltage of 80 volts. After staining with ethidium bromide, DNA was observed and photographed under ultraviolet (UV) light in a document gel apparatus. The existence of a high molecular weight band without gel elongation was considered as a criterion for DNA quality (10).

Primers: Specific primers prepared from Sinagen Company were used for polymerase chain reaction experiments. The primers were freeze-dried in the tube and diluted with sterile double-distilled water to initial concentrations. The diluted primers were then kept at -20$^{\circ C}$ until using. The sequences of used primers and PCR conditions are shown in Tables 1 and 2, respectively (10).

Table 1- Primers' properties and PCR conditions used in this study

Target gene	Primer name	Primer sequence	PCR conditions	Amplicon size (bp)
16S rRNA	fD1 rD1	5′-AGAGTTTGATCCTGGCTCAG-3′ 5′-AAGGAGGTGATCCAGCC-3′	94 °C 3 min; 35 cycles (94 °C 1 min, 55 °C 1.5 min and 72 °C 2 min); 72 °C 10 min.	1500 bp

The reaction mixture for PCR:

Table 2- Preparation of the base solution for specific primers to perform the PCR reaction

Material	Base concentration	Final concentration	Added value
Double sterilized water	-	-	17.5 µl
PCR buffer	10 X	1 X	2.5 µl
$MgCl_2$	50 mM	2.5 mM	2 µl
dNTPs	10 mM	./2 mM	./5 µl
Reverse1 primer	10 pmol/µl	50 pmol	1 µl
Forward2 primer	10 pmol/µl	50 pmol	1 µl
DNA polymerase	5 unit/µl	2.5 Unit	./5 µl
Total volume	-	-	25 µl

Results

In this study, a total of 130 strains were isolated from infected diseased apricot samples, collected from Hamedan province. Based on the color, type of colonies on the culture medium (Figure 1), and the electrophorized total soluble protein pattern of the bacterial strains, 65 strains were selected as a representative for further investigation (Table 3).

The hypersensitivity reaction was performed on the geranium leaves which 48 to 72 hours about 30 strains showed symptoms of hypersensitivity (Figure 2).

Based on the pathogenicity test, two weeks after inoculation, the symptoms of cankers appeared as necrotic spots, which eventually led to the drying of the leaves (Figure 3). Among the 30 representative's strains, 27 strains were Gram negative and three strains were Gram positive.

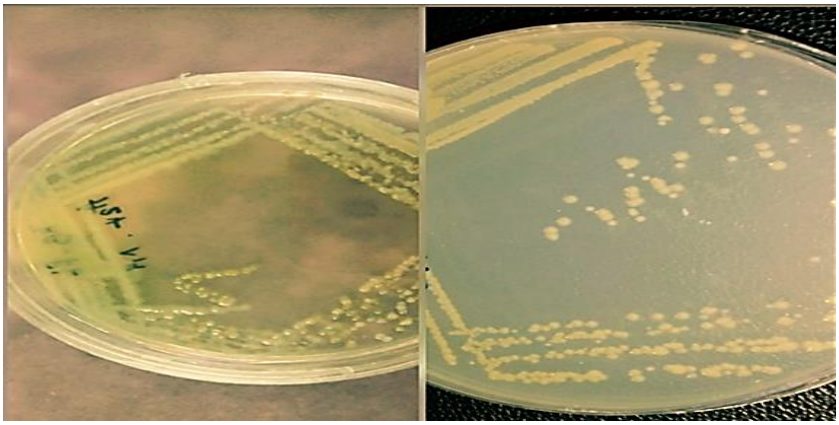

Fig. 1- Bacterial colonies isolated from apricot canker on King's B medium

Table 3- Places and names of the bacterial strains obtained from infected apricot trees in Hamedan province

Sampling place	Strain name
Hamedan	FB1, FB2, FB3, FB4, FB5, FB6, FB7, FB8, FB9, FB10, FB11, FB12, FB13, FB14, FB15, FB16, FB17, FB18, FB19, FB20, FB61, FB62, FB63, FB64, FB65
Malayer(Avarzaman)	FB31, FB32, FB33, FB34, FB35, FB36, FB37, FB38, FB39, FB40
Nahavand	FB41, FB42, FB43, FB44, FB45, FB46, FB47, FB48, FB49, FB50
Tuyserkan(Serkan)	FB51, FB52, FB53, FB54, FB55, FB56, FB57, FB58, FB59, FB60
Asadabad	FB21, FB22, FB23, FB24, FB25, FB26, FB27, FB28, FB29, FB30

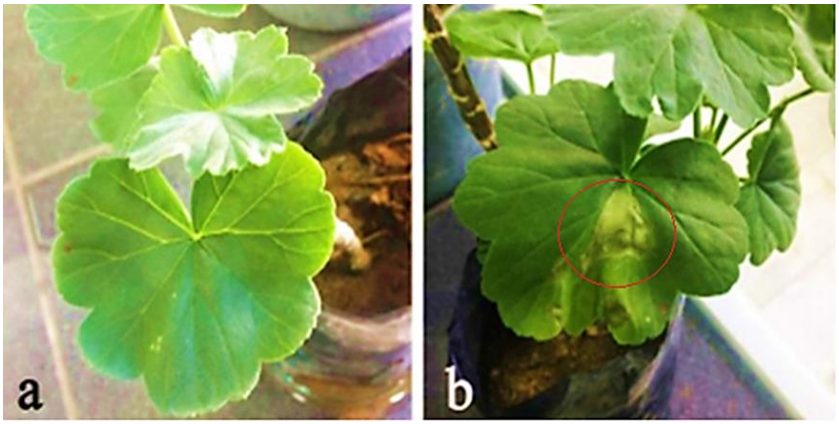

Fig. 2- Hypersensitivity reaction on geranium leaves, a) Negative control, b) Leaves inoculated with pathogenic strains isolated from apricot trees

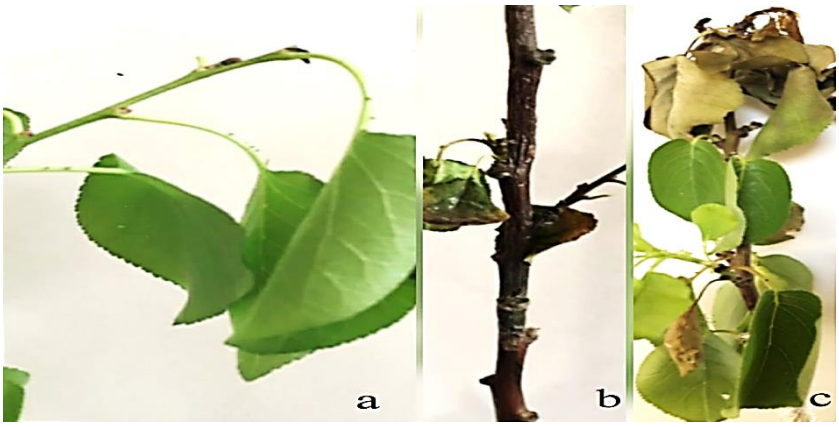

Fig. 3- Pathogenicity of the bacterial strains isolated from apricot seedling. a) Healthy seedlings, b) Infected seedlings by *Pseudomonas syringae*, c) Infected seedlings by *Pantoea agglomerans*

Soluble bacterial cell total protein pattern by SDS-PAGE: A total of 30 representatives strain selected based on their HR on geranium leaves were tested for comparison of their total cell soluble proteins by SDS-PAGE. In some cases, there showed differences and finally they were divided into seven groups (Table 4).

Table 4- Grouping of the bacterial causing apricot canker disease based on their total cell soluble proteins pattern (SAD-PAGE)

Agent Strain	Group members	Group name
FB17	FB8, FB17, FB19, FB20, FB21, FB22	1
FB26	FB24, FB26, FB30	2
FB38	FB32, FB34, FB35, FB38, FB39, FB50, FB54	3
FB42	FB36, FB42	4
FB46	FB44, FB45, FB46, FB48, FB49, FB55, FB59, FB60	5
FB61	FB61	6
FB5	FB5, FB27, FB31	7

Phenotypic characteristics of the tested bacterial strains: Following performing pathogenicity test, for identification of the strains that induced disease symptoms on apricot seedlings, they were subjected to standard bacteriological tests to characterize their phenotypic features. Results are presented in Table 5.

Table 5- Phenotypic characteristics of pathogenic strains isolated from infected apricot trees

Test	Main group 1 including FB46 as a sequenced representative	Main group 2 including FB61 as a sequenced representative
Gram reaction	-	-
Production of fluorescent pigments	-	+
Hypersensitivity test	+	+
Levan formation	-	+
Oxidase reaction	-	-
Catalase reaction	+	+
Pectolytic capability	-	-
O/F test	F	O
Arginine dihydrolase	+	-
Gelatin hydrolysis	-	+
Starch hydrolysis	-	+
Nitrate reduction	-	+
Growth at 37 C	+	-
Growth at 41 C	-	-
Growth in NaCl 6%	+	+
Growth in NaCl 7%	-	-
Sucrose	+	+
Fructose	+	+
Sorbitol	-	-
Inositol	+	+
Glucose	+	+
Raffinose	+	+
Galactose	+	+
Maltose	+	-
Production of yellow colonies on YDCA	+	-

According to the phenotypic characteristics of the tested bacterial strains (Table 5) they were two main groups which first groups including strain FB61 were identified as *P. syringae* and the second group including strain FB46 were identified as *Pantoea agglomerans*. It is worthy to note that according to literature this is the first report of isolation of *P syringae.* from Hamedan province as a causative agent of apricot canker disease and also the presence of *Pantoea agglomerans* as another agent of this disease worldwide is the first time.

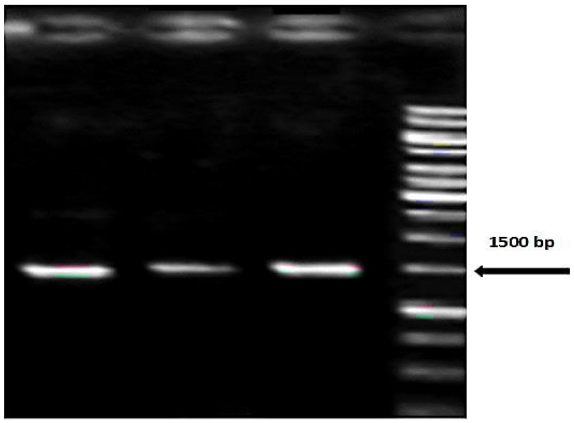

Fig. 4- Amplified 16S rRNA encoding gene using PCR from *Pseudomonas syringae* strains isolated from apricot trees in electrophorized agarose gel 1%.

Polymerase chain reaction (PCR)

16S rRNA encoding gene amplification: Polymerase chain reaction using RD1 and FD1 primers resulted in expected DNA bands in FB46 and FB61 strains (Figure 4). The PCR product of FB46 and FB61 was sent for sequencing to Topazgen Company.

Obtained sequences from two representatives bacterial strains were aligned in the NCBI database by Blast software. Blast results show that strain FB46 has most similarity (95.33%) to *Pantoea agglomerans* RSG19 and FB61 has the most similarity (98.7%) to *Pseudomonas syringae* NCPPB281 (Figure 5).

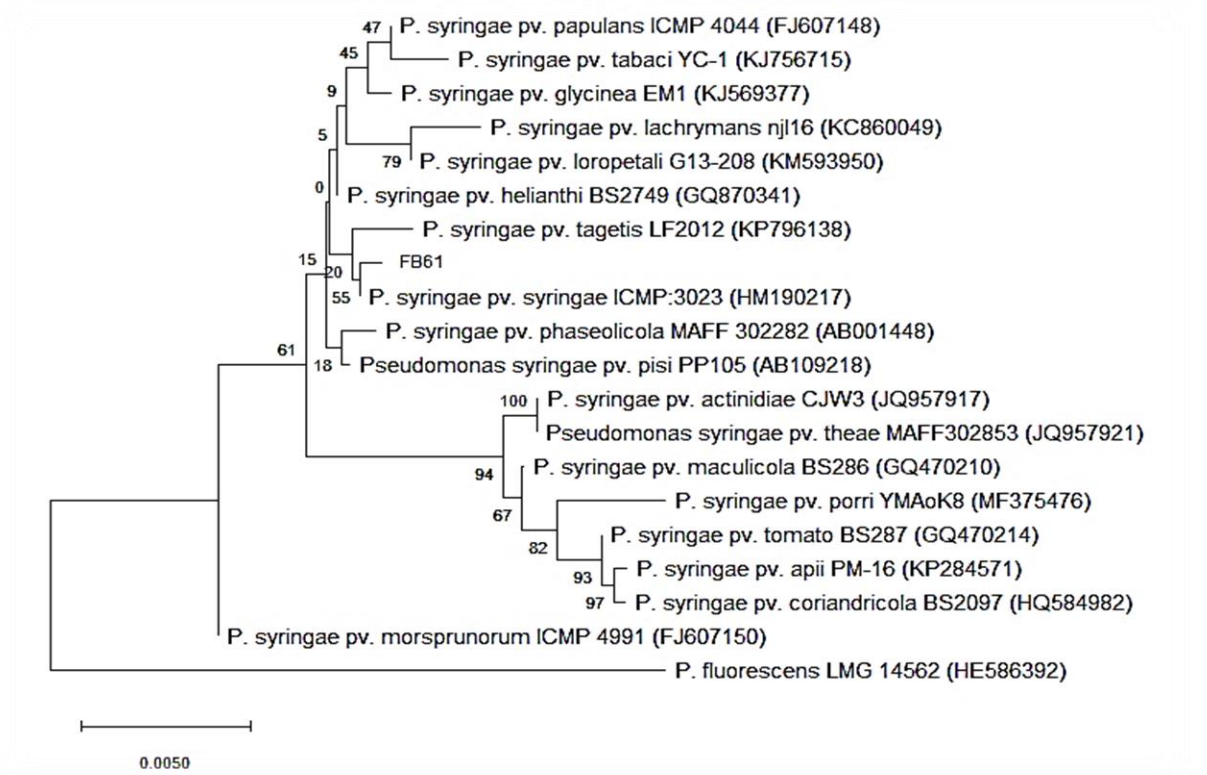

Fig. 5- Phylogenetic tree of strain *Pseudomonas syringae* strain FB61 based on aligned concatenated sequences of the 16S rRNA, *Pseudomonas fluorescens* LMG 14562 (HE586392) selected as out group

Discussion and Conclusion

Among the 130 bacterial strains isolated from affected apricot collected from different regions of Hamedan province, some strains were identified as *P. syringae.* According to HR test on geranium leaves, some strains showed hypersensitivity symptoms by producing necrotic and burned spots on the leaves. Genotypic and pathogenic characteristics of *Pseudomonas* strains isolated from apricot trees in hamedan province using FD1 and RD1 primers and electrophoresis of PCR product on agarose gel resulted 1500 bp DNA band. Sequences of the PCR product showed nearly 98.7% similarity to *P. syringae* .The results obtained are in agreement with of Vasebi *et al*. (11). They isolated *P. syringae* as a causative agent of apricot canker in East Azerbaijan province. They reported among 103 Gram negative strains isolated from different regions of East Azerbaijan province, five strains were identified as *Pss* based on LOPAT and GATTa tests, also the results of the gene BLAST at the NCBI site showed that the strains were 99% similar to *Pseudomonas syringae*. Lelliott *et al*. (12) showed that among 15 defining tests for differentiation of plant pathogen *Pseudomonas*, LOPAT group tests could detect five groups of fluorescent *Pseudomonas*. The researchers used phenotypic and genomic tests for the identification of *P. s.* pv. *syringae* and *P. s.* pv. *morspronorum* (13, 14, 15, 16). In the present study, according to the phenotypic features of the tested bacterial strains isolated from apricot canker disease and their sequences DATA, there were two main groups of strains which the larger group identified as *P. syringae* and the second group as *P. agglomerans.* Results of molecular studies supported the DATA from biochemical and physiological studies which showed that bacterial canker disease of apricot trees in Hamedan province caused by *P. syringae* and *Pantoea agglomerans*. Many researches have shown that the bacterium *P. syringae* has been reported as a causal agent of bacterial canker disease on apricot and some other stone fruits trees (17, 18, 19, 20). As Hamedan province has very cold to freezing days during the winter season, this may damage the apricot young twigs and cause penetration of pathogenic bacteria. Beside this some bacterial strains including *P. syringae* and *P. agglomerans* have been reported to have ice nucleation activity. These pathogenic bacteria later in the spring season will develop the symptoms and cause more damage to the host plants, which provides a good time for sampling (21). We also identified *P. agglomerans,* as one of the causative agents of apricot canker disease. This bacterium previously was reported as one of the agents associated with stone fruits, pome fruits, and walnut trees in Alborz province (21). Taheri *et al*. (22) studied the identification and comparison of phenotypic and genotypic factors of ice-nucleating bacteria. They showed that six percent of the tested *P. agglomerans* strains has ice nucleation activity. It is worthy to note that *P. agglomerans* have also been reported as one of the causes of bacterial leaf spot in ornamental plants from Isfahan, Markazi and Khuzestan provinces (23).

In the present study, *P. syringae* is reported for the first time as a causative agent of apricot bacterial canker in Hamedan province. According to our knowledge from literature there is no report of *P. agglomerans* as a causal agent of apricot canker disease, therefor this is the first report from the presence of this bacterium as a causal agent of apricot bacterial canker disease worldwide.

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