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ORIGINAL ARTICLE

Bacterial Species as Causative Agents Involved in Pistachios Dieback in Iran

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K E Y W O R D S

Bacterial pathogens;
Crop damage;
Pathogenicity;
Pistacia vera

A B S T R A C T

Pistachio dieback (DBP) is a significant disease affecting pistachio trees in Iran, and it has emerged as a serious problem in Kerman province in recent years. This study investigates the role of bacteria as causal agents of DBP under laboratory and field conditions. Samples were collected from infected pistachio orchards in Kerman province from 2015 to 2016. The ability of bacterial isolates to induce disease and colonize vascular tissues was studied using various inoculation methods. Identification of isolates was carried out using biochemical and physiological assays, amplification of the 16S rDNA region, and partial analyses of the gyrA gene. A total of 281 bacterial isolates were obtained from infected trees, of which 148 induced a hypersensitivity reaction on tobacco leaves. Among these, 128 isolates were able to colonize vascular tissues in sub-bark inoculations of pistachio branches under laboratory conditions. In field experiments, 24 selected isolates were able to spread in vascular tissues of pistachio branches and twigs using sub-bark and apical inoculation methods, although disease severity varied. *Staphylococcus pasteuri*, *Bacillus pumilus*, *Bacillus sp*., *Acinetobacter radioresistens*, *Xanthomonas sp*., *Curtobacterium flaccumfaciens*, *Pseudarthrobacter oxydans*, and *Pseudomonas koreensis* were identified as being involved in the dieback of pistachio trees. This work demonstrates that a wide range of bacterial genera and species may be involved in DBP, and urgent strategies should be considered for managing the disease.

Introduction

Pistachio trees produce commercially valuable dry nuts (Eslami *et al*., 2019; Hosseini *et al*., 2022; Nazoori *et al*., 2022a; Nazoori *et al*., 2022b). Die-back of pistachio (DBP) trees (*Pistacia vera*) is one of the most severe diseases, causing significant crop damage annually. In Iran, DBP was first observed in Kerman province (Aminaee and Ershad, 1987). The symptoms of DBP have been observed in at least 85% of pistachio-producing areas in Iran (Heidarian *et al*., 2018). Pathogenic infections affect various parts of

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the tree, such as the canopy and trunk, to different degrees. Infected trees generally exhibit slow canopy growth and fail to undergo reproductive growth. In severe cases, the annual growth of shoots is also reduced. The initial symptoms of DBP appear as small areas of brown to black discoloration on the surface of infected twigs and branches. These symptoms quickly spread in all directions, both longitudinally within vessels and piths and radially within parenchyma. The disease can be recognized by the distinct discolorations between infected and healthy tissues. The color of infected bark and wood tissues changes to dark brown and black. The margins of infected areas on the bark are distinguishable from the healthy bark based on color changes and sometimes morphological changes such as flattening. Generally, DBP progresses from the top of the tree downwards (Alizadeh *et al*., 2000; Ghelichi *et al*., 2012; Aminaee and Ershad, 1987). The infection is perennial, and in severely infected trees, DBP will infect and kill branches over the course of some years.

In recent years, DBP has become a major concern due to a significant decrease in pistachio production, especially under poor orchard management. Several studies have reported on the role of biotic factors in the occurrence of DBP. In Greece, *Eutypa lata* was isolated from infected pistachio branches and confirmed as a new host (Rumbos, 1986). *Botryosphaeria dothidea* has been identified as the causal agent of pistachio shoot blight in California (Michailides and Ogawa, 1985). *Paecilomyces variotii* was reported to be associated with DBP (Aminaee and Ershad, 1987). Alizadeh *et al*. (2000) identified *P. variotii*, *Cytospora* sp., and *Natrassia mangiferae* as the causal agents of DBP in Kerman province (Alizadeh *et al*., 2000). *Neoscytalidium dimidiatum* has been isolated from the roots and stems of pistachio trees as the causal agent of pistachio die-back in Turkey (Kurt *et al*., 2019). In recent years, in addition to fungal pathogens, bacterial agents have also been reported to be involved in DBP of pistachio (Baradaran and Ghasemi, 2010). Bacterial die-back of pistachio has been associated with *Xanthomonas* (Edwards and Taylor, 1998). Later, the causal agent was identified as *Xanthomonas translucens* (Facelli *et al*., 2002; 2005). Subsequent studies investigated the diversity among *Xanthomonas* isolated from pistachio in Australia (Marefat *et al*., 2006). *Xanthomonas* sp. from cankers and leaf spots on 1-year-old pistachio seedlings were subjected to biochemical and physiological tests and identified as a causal agent (Tarighi and Rahimian, 2001).

The etiology of DBP is still unknown, and further studies are required to explore different pathogenesis scenarios. Given the severity of DBP in pistachio orchards in Kerman province and the complexity of the disease, this study focused on bacterial agents associated with the disease. The aim was to identify novel, previously undescribed bacterial pathogens involved in pistachio dieback in Kerman province, Iran.

Material and Methods

Sample collection and isolation of bacteria

Twigs and branches (on average 50-100 cm in length) with die-back symptoms were collected from Rafsanjan, Kerman, Zarand, Anar, Shahrbabak and Sirjan counties from 38, 10, 14, 6, 3 and 6 orchards, respectively, during January 2015 to December 2016. In each orchard a representative sample from different trees with clear DBP symptoms was collected. Samples were immediately kept on ice and cooled until transferred to the laboratory for isolation. In the laboratory, the infected twigs and branches were cut in 5 cm long pieces. From each cut, 5×5 mm cross-sections from the border of healthy and infected tissues or from areas with no visual symptoms were prepared. The sections were washed with distilled water, then disinfected in 70% ethanol for 30 seconds and rinsed three times with sterile distilled water. The sections were transferred into tubes (Maxwell, Ningbo Fuchun Co., China) containing 5ml of 0.85% sterile NaCl solution. The sample suspensions were kept at

room temperature for 15 min, then vortex and 1 ml from each suspension was streaked on nutrient agar plus 5% (w/v) sucrose (NAS) and yeast dextrose carbonate (YDC) agar media. The plates were incubated at 28°C in the dark for 7 days. The plates were examined daily for possible growth. Single colonies were selected and re-cultured on NAS medium. The purified isolates were cultured on nutrient agar (NA) slants as working cultures and nutrient broth (NB) containing 30% glycerol at -70°C for long time storage (Schaad *et al*., 2001). All bacterial isolates were subjected to hypersensitivity reactions. The ability of the isolates to induce hypersensitivity responses were assessed on geranium plants (*Pelargonium hortorum*). The purified isolates were grown on NA for 24-48 h at 28°C, and then suspensions of ~10^7 CFU/ml of each isolates in 2 ml of deionized water were prepared. Hypodermic syringes fitted with a fine needle were used to infiltrate suspension of bacteria into the intercellular spaces of geranium leaves (Ocho, 2006; Klement *et al*., 1990). Three leaves were infiltrated by each isolate. Infiltrating leaves with sterile distilled water serve as a negative control. The hypersensitivity reactions were evaluated within 24-48h post inoculations compared with negative control under the greenhouse conditions.

Pathogenicity tests

Pathogenicity tests were carried out for bacterial isolates in laboratory and field experiments on *P. vera* cv. *Fandoghi*, which was most susceptible to die-back of pistachio.

Lab

The ability of 148 selected bacterial isolates to produce disease was singly assessed through two inoculation methods. In the first method, two-year old, healthy twigs (30 cm length) were collected from mature trees (*P. vera*). The twigs were assessed for any vascular discolorations before using in the experiments. A 24h-old culture of bacterial isolates was used to produce a suspension in sterile potassium phosphate buffer containing 10^7 CFU/ml. The twigs were surface-disinfected, as described above, before inoculations. One hundred microliter inoculum was injected beneath the inner bark of twig using new sterile syringes for each isolate. The inoculation sites were covered with paraffin film (Parafilm, Bemis, USA) for the first two days, and then removed. The treated twigs were singly placed upright in sterile tubes containing moist cotton plug to prevent from drying out the twigs. The sterile tubes were incubated at 28°C in the dark for 20 days. Experiments were carried out with three replicates for each bacterial isolate. Sterile-distilled water was used as control. The second method was similar to the first but bacterial suspensions were injected into the xylem vascular tissue under the apical buds of the twigs.

Pathogenicity of the isolates in both methods was evaluated 20 days after inoculations. The establishment of the bacterial pathogens and the progression of symptoms were also evaluated through re-isolation of bacteria from the margin between discolored and non-discolored infected vascular tissues. The morphological and physiological properties of pure culture of re-isolated bacteria were compared with those pure cultures used for inoculations. Based on the lesion extension and vascular discolorations from the point of inoculation on twigs and branches using a millimeter ruler, bacterial isolates were categorized into three groups, 1: 0.5-3.5 cm; 2: 3.6-6.6 cm and 3: 6.7-10 cm.

Field

Based on pathogenicity tests conducted in the laboratory, 24 bacterial isolates were selected for further evaluations under in vivo tests. During 2016-2018, experiments were conducted in late winter and early spring on healthy two year lignified twigs of 30-year old pistachio trees (*P. vera* cv. *Fandoghi*), at the Iranian Pistachio Research Center's orchard, using two inoculation methods. In the first method, a 24h-old bacterial culture was used to prepare a fresh

suspension with a concentration of 10^7 CFU/ml. The suspension was injected beneath of the inner bark of two-year-old pistachio twigs using sterile syringes for each isolate. In the second method, the bacterial suspensions were injected into the xylem vascular tissue under the apical buds of the twigs. In both methods, before injection, the target area was washed and disinfected with 70% ethanol and the injected areas were sealed with paraffin film. There were two blocks (tree), each with three twigs per bacterial isolates. Control twigs were treated with sterilized distilled water and processed the same way as inoculated twigs.

Disease severity was assessed based on the vascular longitudinal and radial discolorations 2 and 12 months after inoculations. Longitudinal stripes were revealed by detaching the bark with a knife. For the measurement of symptoms, stems were longitudinally cut using a knife and wood discoloration appearing in the outer or inner xylem vessel around the inoculation site were determined both upward and downward using a millimeter ruler. The establishment of the bacterial pathogens and the progression of symptoms as well as morphological and physiological properties of re-isolated bacteria were evaluated as described in Lab experiments.

Phenotypic characteristics of selected bacteria

The phenotypic characteristics of 24 selected bacterial isolates with the highest ability to produce disease and develop in vascular tissues of twigs or branches *in vitro* and *in vivo* were examined through gram reaction, O/F test, levan production, Kovac oxidase test, potato soft rot, arginine dihydrolase activity, fluorescence on KB medium, catalase activity, tween 80, casein, starch and gelatin hydrolysis and nitrate reduction tests (Lelliott and Stead, 1987; Schaad and Jones, 2001).

Molecular identification

For this, one colony from a 24h old NA culture was used for DNA extraction. The DNA was extracted using a DNP kit (Sinaclon, Tehran, Iran). The extracted DNA was stored at -20°C as a template for PCR amplification of 16SrDNA and *gyrA* genes using the primers presented in Table 1. The PCR products were analyzed on 1% agarose gel (w/v). The PCR products were sent to Bionner Company (South Korea) for sequencing. The sequences obtained were further aligned to other closely related bacterial species deposited in NCBI database using the BlastN program (https://blast.ncbi.nlm.nih.gov).

Phylogenetic trees was constructed using the Neighbor-joining and Maximum-likelihood methods (Saitou and Nei 1987; Tamura *et al*., 2013) based on distance matrix data. The evolutionary distances were calculated using the Jukes-Cantor model (Jukes and Cantor, 1969). Boot-strap values (1000 replicates) were calculated to validate the reproducibility of the branching pattern of the tree. The sequences analyses were conducted with MEGA 6 software (Tamura *et al*., 2013).

Statistical analysis

The average values of vascular discoloration ratings were separately determined for each twig after 20 days, two and twelve months after inoculations. The effects of inoculation methods, bacterial isolates, and their interactions on the extend of vascular discoloration were analysed using SPSS statistics software (Version 16.0, IBM Corp) by univariate analysis (ANOVA). Mean comparisons were made using Duncan's new multiple range test at 5 % probability.

Table 1. Primers used for sequencing of bacterial isolates.

Primer	Amplicon	Target	Sequence	PCR Program	Isolates	Source
fD1 **rD1**	1500 bp	16SrDNA 16SrDNA	5′-AGAGTTTGATCCTGGCTCAG-3′ 5′-AAGGAGGTGATCCAGCC-3′	94, 9 min 94, 30 s 56, 30 s × 30 72, 90 s 72, 10 min	RK1315, ZD1415, KE4515, RP17315, RRO16415, KA1415, RRO6515, RJ10316, RJ12415, KE2516, RK2515, RL11415, RL14415, RM7415, RM13316, RRO3415, KA3415	Weisburg *et al*., 1991
63f **1387r**	1300 bp	16SrDNA 16SrDNA	5′-CAGGCCTAACACATGCAAGTC-3′ 5′-GGGCGGWGTGTACAAGGC-3′	94, 4 min 94, 60 s 65, 45 s × 40 72, 60 s 72, 5 min	AH1615, RH18516, RR4715, RR8516, RR15516, RR9615, RR5516	Marchesi *et al*., 1998
***gyrA*-42f** ***gyrA*-1066r**	928 bp	*gyrA* *gyrA*	5′-CAG TCAGGA AAT GCG TAC GTC CTT-3′ 5′-CAA GGT AAT GCT CCA GGC ATT GCT-3′	94, 4 min 94, 30 s 60, 30 s × 35 72, 1 min	KA1415, KA3415, RL14415, RM7415	Rooney *et al*., 2009

Results

Pistachio dieback symptoms

The initial symptoms of DBP appeared in form of small areas (less than one centimeter) of brown to black discolorations on the surface of the bark in infected twigs and branches (Fig. 2 a-c).

In infected orchards, all aerial parts of trees such as canopy and trunk and their components were affected although to varying degrees.

Symptom development began in the middle of spring and progressed throughout the summer. Hot, dry weather followed by high yields made symptoms worse. Generally, infected trees showed a slow growing canopy and failed to produce pistachios in the year following initial infection. In severe infections, the annual growth of shoots was reduced, or no growth occurred (Figure 1). The initial symptoms appeared in twigs or branches, but eventually spread downward to the stems and the trunk of the tree.

Fig. 1. Symptoms of die-back on affected pistachio trees (*Pistacia vera* cv. *Fandoghi*) under field conditions. a, sparse canopy tree with about 30 years old at the end of May; b, reduced annual shoot growth at the end of Autumn; c and d, dieback of pistachio branches with about 25 years old (*Pistacia vera* cv. *Kaleh-Ghouchi*)

Symptoms spread from the point of infection in all directions; longitudinally within vessels and piths and radially within parenchymatic tissue. In heavily infected twigs or branches almost the entire bark showed brown to black discoloration. Culturing cross and longitudinal sections in culture media revealed that bacterial colonization of stems, branches or the trunk which characterized by sunken lesions in the wood (Figure 2 d-e). Disease progression was recognizable due to the distinguished discolorations between infected and healthy tissues. On the surface of bark, the margins of infection areas could be distinguished from healthy bark based on the changing of color and sometimes flattened lesions (Fig. 2 f-h). In general, DBP progressed from the top of the tree downwards. Infection was perennial, and in severely infected trees, DBP killed branches over 2-3 years. The discolored areas of the outer tissue or bark became dried out, shriveled and cracked. The pattern of discolored areas in cross and longitudinal sections showed that in upward tissues the colonization covered all vascular tissues and in downward sections the discoloration areas were linear (Figure 3).

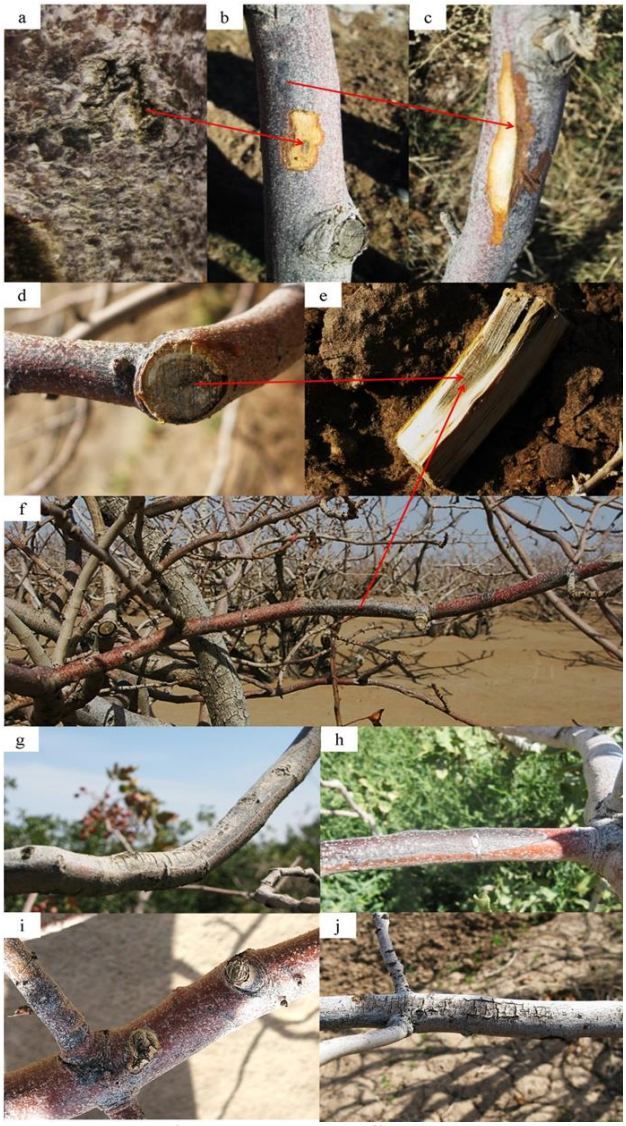

Fig. 2. Initial infection and symptom development of pistachio die-back on affected pistachio trees (*Pistacia vera* cv. *Fandoghi*). a, wounds caused by abiotic factors; b, small lesion on the bark and cambium; c, d, cross- and longitudinal clear boundary between infected and healthy tissues due to the distinguished discolorations; f-j, visible symptoms on surface of bark.

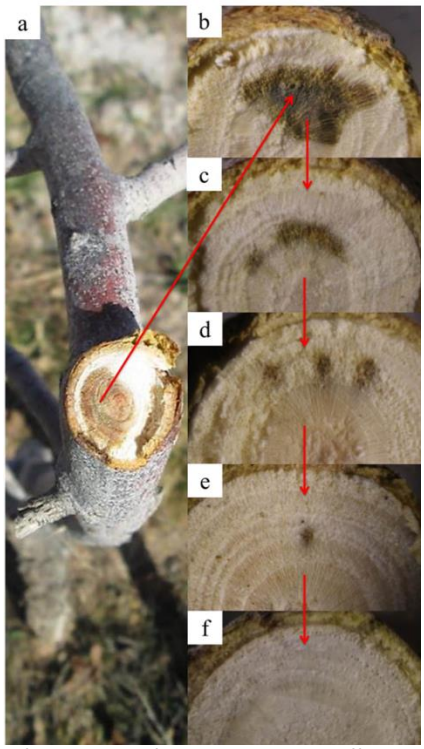

Fig. 3. Sequential cross-sections in xylem vessels of (1 cm, thick) of pistachio die-back on affected trees (*Pistacia vera* cv. *Fandoghi*) cut distally. a, a lesion and vascular discoloration on secondary branch; b-f, pattern of decreasing discolored areas upward to downward of branches with a length of 20 cm.

The observed symptoms of the infected tissues could grouped as: (1) general symptoms: brown to black wood discoloration on parts or all of the wood tissue; (2) local symptoms: brown to black spots at the cross-sections; (3) no visual symptoms in vesicular tissues; (4) dark brown to black discoloration symptoms only under bark when cut longitudinally; (5) violet-colored spots and (6) ring-shaped discoloration at the cross-section of the branches (Figure 4).

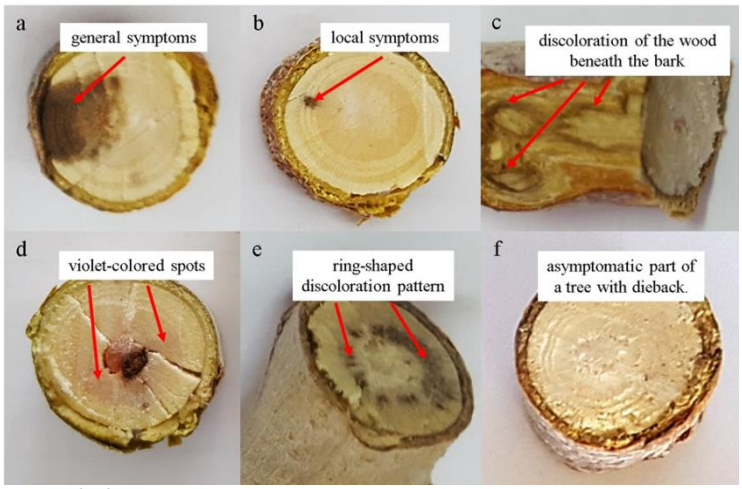

Fig. 4. Cross-sectional symptoms of infected pistachio trees with die-back a) general symptoms, b) local symptoms, c) discoloration of the wood beneath the bark, d) violet-colored spots, e) ring-shaped discoloration pattern and f) asymptomatic part of a tree with dieback.

Both bacteria and fungi were isolated from diseased samples exhibiting symptoms of type 1 and 4, whereas bacterial agents were more abundant in symptom types of 2, 3, 5 and 6.

Bacterial isolation

Overall, 77 samples were collected from infected pistachio orchards with different DBP symptoms. Out of 77 samples, bacterial isolates were isolated from 51

samples, while in 26 samples no bacterial isolates could be detected. A total of 281 bacterial isolates were obtained from Rafsanjan (142), Anar (43), Zarand (28), Sirjan (39), Kerman (18) and Shahrbabak (11) regions. Bacterial isolates were isolated from most samples collected during February to May. While the samples collected during the months of June to September (dry season) were either negative or it was difficult to isolate the bacterial strains. The most frequent isolates belonged to *Bacillus*, *Curtobacterium*, *Staphylococcus*, *Pseudomonas*, *Xanthomonas*, *Acinetobacter* and *Pseudarthrobacter* with a frequency of 52%, 16%, 12%, 8%, 7%, 3%, 2%, respectively.

Hypersensitivity reaction test (HRT)

The results of hypersensitivity reactions on tobacco leaves showed that out of 281 bacterial isolates, 148 were positive in these assays after 48h. The positive bacterial isolates were subjected to pathogenicity assays under laboratory and field conditions.

In vitro tests

Overall, out of 148 bacterial isolates, 128 were able to cause disease although to different degrees. The means of lesion extension of bacterial isolates on inoculated twigs ranged from 0.5 to 8.5 cm. The highest disease progress was recorded from isolate KA3415 with a 9.5 cm longitudinal extension.

Based on mean lesion extension from the point of inoculation on twigs, the 128 bacterial isolates were categorized in three groups.

Lesion extension in first, second and third group was 0.5 to 3.5, 3.6 to 6.6 and 6.7 to 10 cm, respectively. The frequency of the isolates was 71.9%, 19.5% and 8.6 % for group 1, 2 and 3, respectively.

Based on the results, 24 bacterial isolates with the highest ability to thrive in twigs were studied more intensively. Significant differences were observed between the bacterial isolates in term of radial and longitudinal development on two year inoculated twigs. The highest lesion extension was recorded in KA3415 then followed by RR9615, RL14415, RM7415, RJ12415, AH1615 and RM13316 isolates in third group 3, although no significant differences were found (Figure 5). In control twig vascular discoloration was observed closed to injection points, but isolation yielded no bacteria.

In all cases, the results demonstrated the presence of bacterial isolates in the lesions either from inoculation sites or from the border of infected and healthy tissues (Figure 6). Although staining of wood was observed in control twigs closed to injection point, but no bacteria were isolated.

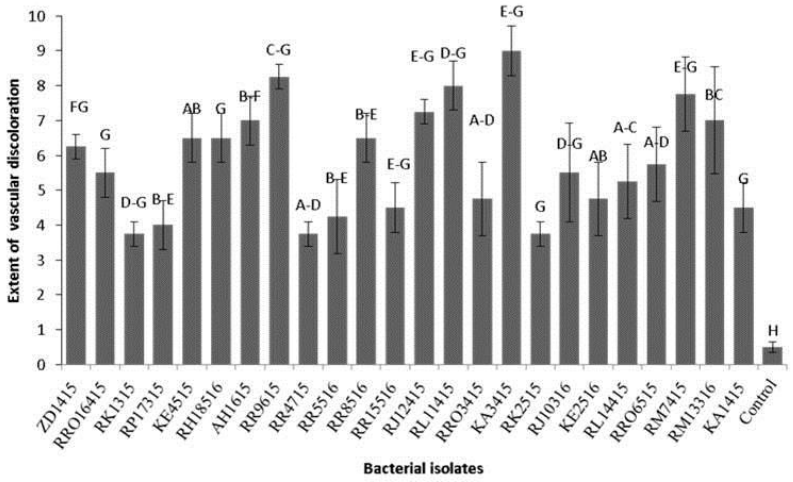

Fig. 5. Vascular discolorations (centimeters, CM) of pistachio twigs after sub-bark inoculations (*Pistacia vera* cv. *Fandoghi*) with bacterial isolates after 20 days.
* Different Uppercase letters over bars indicate significant differences among means in vascular discolorations ($p < 0.05$) by Duncan's new multiple range test.

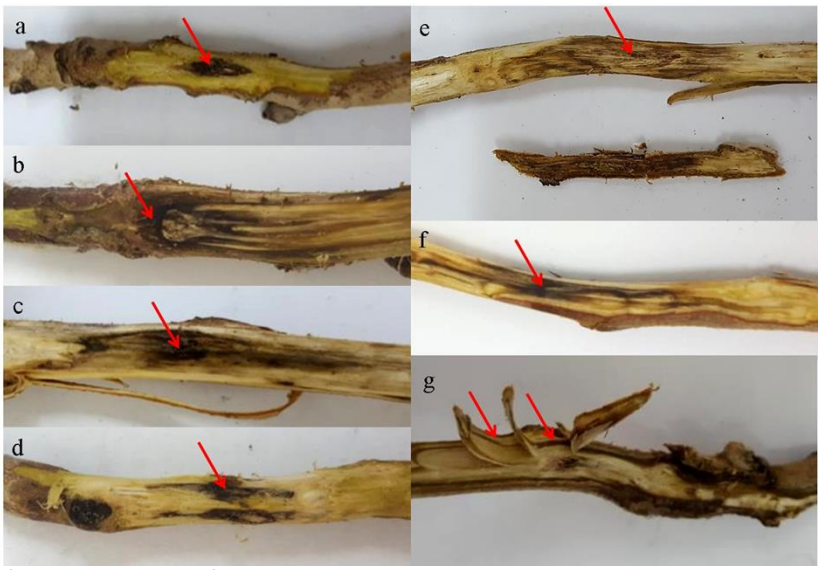

Fig. 6. The symptoms of vascular discoloration of two-year pistachio twigs inoculated with selected bacterial isolates. a) no-inoculations control; b, c, d, e, f) inoculated with bacterial isolates; Arrows show the point of inoculations. g, visible colonization sunken-in vascular tissues.

Field studies

The symptoms of the disease for sub-bark inoculations with 24 selected bacterial isolates showed longitudinally infections radiating up- and downward from the point of inoculation with a higher prevalence toward the trunk of the trees. For apical inoculations, discoloration in the area of injection covered the entire cross-section of the branch and then progressed linearly towards the main branch and trunk of the tree (Fig. 7). The two inoculation methods under orchard conditions differed in terms of the longitudinal development of the bacteria and extension of symptoms. In sub-bark inoculations longitudinal lesion development on the branches were higher than those of apical inoculations.

Similar to the in-vitro tests, the phenotypic characteristics and hypersensitivity reaction of isolated bacterial from inoculated twigs were the same as original ones.

Fig. 7. The symptom of pistachio die-back in inoculations with bacterial isolates under field conditions after one year. Die-back of branches in sub-bark (a) and apical (b) inoculations; vascular longitudinal and radial discolorations in sub-bark (c) and apical inoculations (d), respectively; the arrows indicate the point of inoculations; Sequential cross-sections in xylem vessels (1 cm, thick, E-I) in inoculated twigs represent longitudinal development of discoloration downward of secondary braches (*Pistacia vera* cv. *Fandoghi*).

Severity of disease symptoms as described in figure 8 from the two inoculation methods two and 12 months after inoculations differed statistically (Table 2). Progress was clearly visible on the pistachio trees four weeks after inoculations. The highest vascular discolorations were observed for isolates RR9615, RJ12415, RM7415, KA3415 and lowest for isolates AH1615, RR8516, RP17315 in both inoculation methods (Fig.s 9 and 10). On average, the lowest and highest discolorations after 2 months were 3 to 13.75 cm for sub-bark (method 1) and 2 to 13 cm for apical inoculations (method 2). After one year the discolorations ranged from 5 to 24 cm (method 1) and 4.5 to 18 cm (method 2), respectively. Generally, the length of wood discoloration in the apical inoculation method varied from 1 to 14 cm after 2 months and 3.5 to 20.5 cm after 12 months. For sub-bark inoculation, the length of discoloration varied from 2 to 14.5 cm and 4.5 to 27 cm after 2 and 12 months, respectively. In control twigs inoculated with sterile distilled water resulted 6-15 mm wood staining closed to injection point, but no bacteria was isolated.

Table 2. Analysis of variance for inoculations of two-year old pistachio twigs with selected bacterial isolates under field conditions.

Source	Type III Sum of Squares	df	Mean Square	F	P value
Sub-bark inoculation after 20 days					
Isolates	166.280	24	6.928	8.780	.000
Replication	2.081	1	2.081	2.637	.117
Error	18.939	24	.789		
Total	1766.520	50			
Sub-bark inoculation after two months					
Isolates	686.785	24	28.616	16.462	.000
Block	1.411	1	1.411	.812	.377
Error	41.719	24	1.738		
Total	3368.200	50			
Sub-bark inoculation after twelve months					
Isolates	1897.630	24	79.068	19.490	.000
Block	15.905	1	15.905	3.920	.059
Error	97.365	24	4.057		
Total					
Apical inoculation after two months					
Isolates	607.880	24	25.328	29.145	.000
Block	5.848	1	5.848	6.730	.016
Error	20.857	24	.869		
Total	2792.830	50			
Apical inoculation after twelve months					
Isolates	854.737	24	35.614	19.259	.000
Block	11.424	1	11.424	6.178	.020
Error	44.381	24	1.849		
Total	5478.310	50			
Between two inoculation methods after two months					
Method	12.041	1	12.041	9.316	.004
Isolates	1267.559	24	52.815	40.863	.000
Method × Isolates	27.107	24	1.129	.874	.632
Block	6.503	1	6.503	5.031	.029
Error	63.333	49	1.293		
Total	6161.030	100			
Between two inoculation methods after twelve months					
Method	534.072	1	534.072	184.382	.000
Isolates	2592.711	24	108.030	37.296	.000
Method × Isolates	159.655	24	6.652	2.297	.007
Block	27.144	1	27.144	9.371	.004
Error	141.931	49	2.897		
Total	17542.830	100			

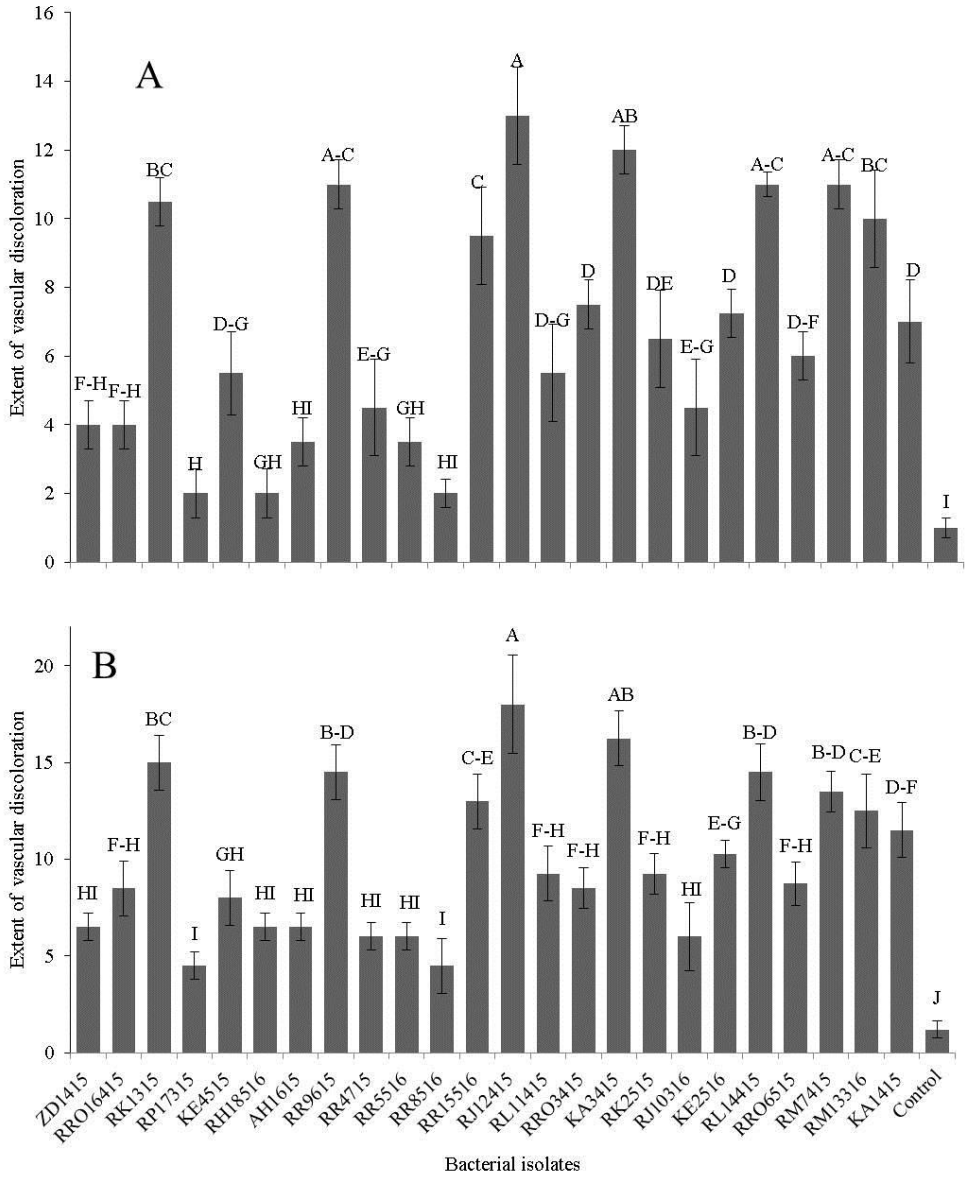

Fig. 8. Vascular discolorations (centimeters) of pistachio wigs after apical inoculations with bacterial isolates on living trees (*Pistacia vera* cv. *Fandoghi*) after two (A) and twelve (B) 12 months

* Different Uppercase letters over bars indicate significant differences among means in vascular discolorations ($p < 0.05$) by Duncan's new multiple range test.

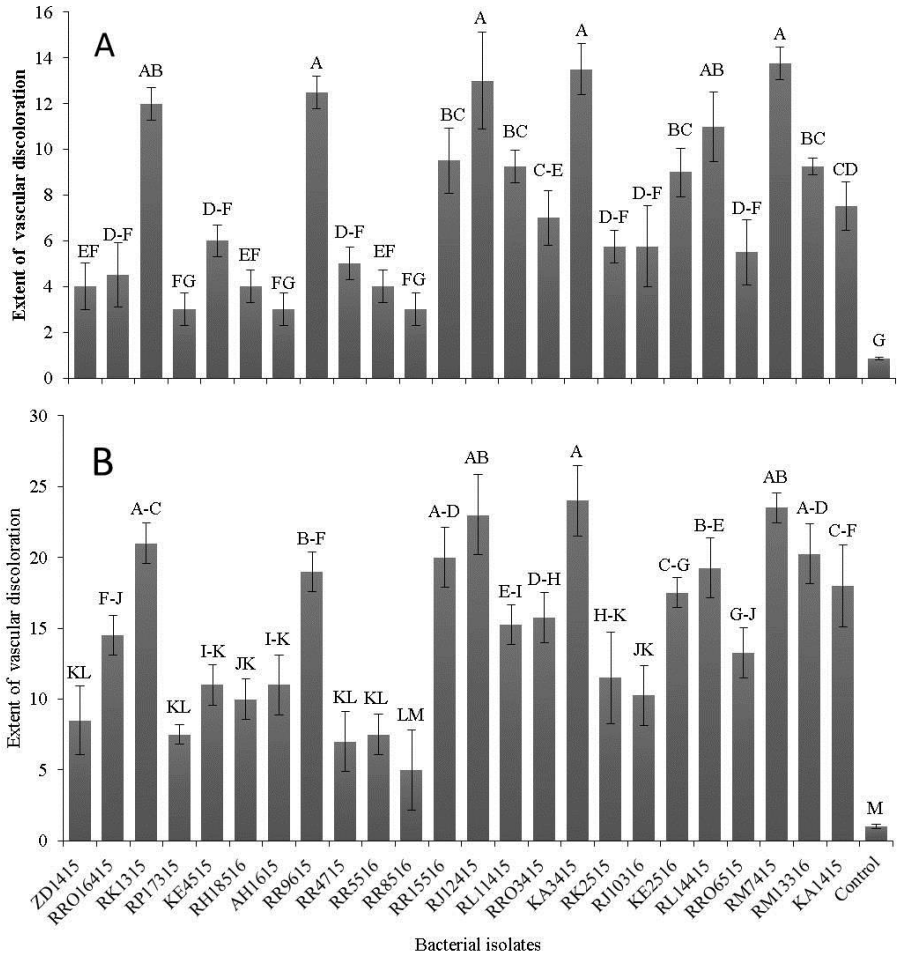

Fig. 9. Vascular discolorations (centimeters) of pistachio branches after sub-bark inoculations with bacterial isolates on living trees (*Pistacia vera* cv. *Fandoghi*) after two (A) and twelve (B) 12 months

* Different uppercase letters over bars indicate significant differences among means in vascular discolorations ($p < 0.05$) by Duncan's new multiple range test

Table 3. Characteristics of selected bacterial isolates associated with pistachio die-back in Kerman Province Iran.

Strain code	Accession numbers 16S rDNA	Genus/Species	Date isolated	Type of [a] symptoms	County of origin
ZD1415	MT355870	*Acinetobacter radioresistens*	April 2015	General	Zarand
RRO16415	MT355872	*Pseudarthrobacter oxydans*	April 2015	No symptom	Rafsanjan
RK1315	MT355844	*Curtobacterium flaccumfaciens*	March 2015	General	Rafsanjan
RP17315	-	*Staphylococcus pasteuri*	March 2015	General	Rafsanjan
KE4515	MT371429	*Staphylococcus pasteuri*	May 2015	Local	Kerman
RH18516	MT371254	*Pseudomonas koreensis*	May 2016	Local	Rafsanjan
AH1615	MT371257	*Pseudomonas koreensis*	June 2015	Local	Anar
RR9615	-	*Xanthomonas* sp.	June 2015	General	Rafsanjan
RR4715	-	*Xanthomonas* sp.	July 2015	General	Rafsanjan
RR5516	-	*Xanthomonas* sp.	May 2016	General	Rafsanjan
RR8516	-	*Xanthomonas* sp.	May 2016	General	Rafsanjan
RR15516	-	*Xanthomonas* sp.	May 2016	General	Rafsanjan
RJ12415	-	*Bacillus* sp.	April 2015	Local	Rafsanjan
RL11415	-	*Bacillus* sp.	April 2015	General	Rafsanjan
RRO3415	-	*Bacillus* sp.	April 2015	General	Rafsanjan
KA3415	MN861985	*Bacillus pumilus*	April 2015	Local	Kerman

RK2515	-	*Bacillus* sp.	May 2015	General	Kerman
RJ10316	-	*Bacillus* sp.	March 2016	General	Rafsanjan
KE2516	-	*Bacillus* sp.	May 2016	General	Kerman
RL14415	MT022519	*Bacillus pumilus*	April 2015	Local	Rafsanjan
RRO6515	-	*Bacillus* sp.	May 2015	General	Rafsanjan
RM7415	MT022520	*Bacillus pumilus*	April 2015	Local	Rafsanjan
RM13316	-	*Bacillus* sp.	March 2016	Local	Rafsanjan
KA1415	-	*Bacillus* sp.	April 2015	Local	Kerman

a: General, brown to black wood discoloration on parts or all of the wood tissue; local, brown to black spots at the cross-sections; no symptom, The bacterial isolates were collected from different orchards in Rafsanjan, Kerman, Zarand, Anar, Shahrbabak and Sirjan counties

(1) general symptoms: brown to black wood discoloration on parts or all of the wood tissue; (2) local symptoms: brown to black spots at the cross-sections; (3) no visual symptoms in vesicular tissues; (4) dark brown to black discoloration symptoms only under bark when cut longitudinally; (5) violet-colored spots and (6) ring-shaped discoloration at the cross-section of the branches. The bacterial isolates were collected from different orchards in each country of origin.

Phenotypic and Molecular identification

The selected pathogenic bacterial isolates (24 isolates) were identified through biochemical, physiological and molecular tests at the species level. *Staphylococcus pasteuri* (RP17315 and KE4515 strains), *Bacillus pumilus* (KA3415, RL14415 and RM7415), *Acinetobacter radioresistens* (ZD1415), *Curtobacterium flaccumfaciens* (RK1315), *Pseudarthrobacter oxydans* (RRO16415) and *Pseudomonas koreensis* (RH18516 and AH1615) were identified with accession numbers of 16SrDNA gene sequences listed in Table 3.

To identify the isolates belonging to the genus of *Bacillus*, a 964 bp fragment of the *gyrA* gene was amplified and sequenced in four isolates. The *gyrA* nucleotide sequence of the KA3415 strain was deposited into GenBank with accession number MT032005.

The results of 16SrDNA sequence similarity score of 12 isolates belonged to *Bacillus* sp. showed a similarity higher than 98% with those strains deposited in GenBank. *GryA* gene sequencing showed the amplification of a fragment with a size of 928 bp only in KA1415, KA3415, RM7415 and RL14415 isolates with a similarity higher than 99% with those *Bacillus pumilus* strains deposited in GenBank. Lack of *gryA* gene amplification in the other 7 isolates may indicate species diversity in this genus or species identification requires combining multiple sequences from different genes to draw a conclusion.

Based on the phylogenetic analysis of 16S rRNA in representative isolates in different species, were clustered with reference strains with high similarity relationships (Supplementary data Figures 10-16).

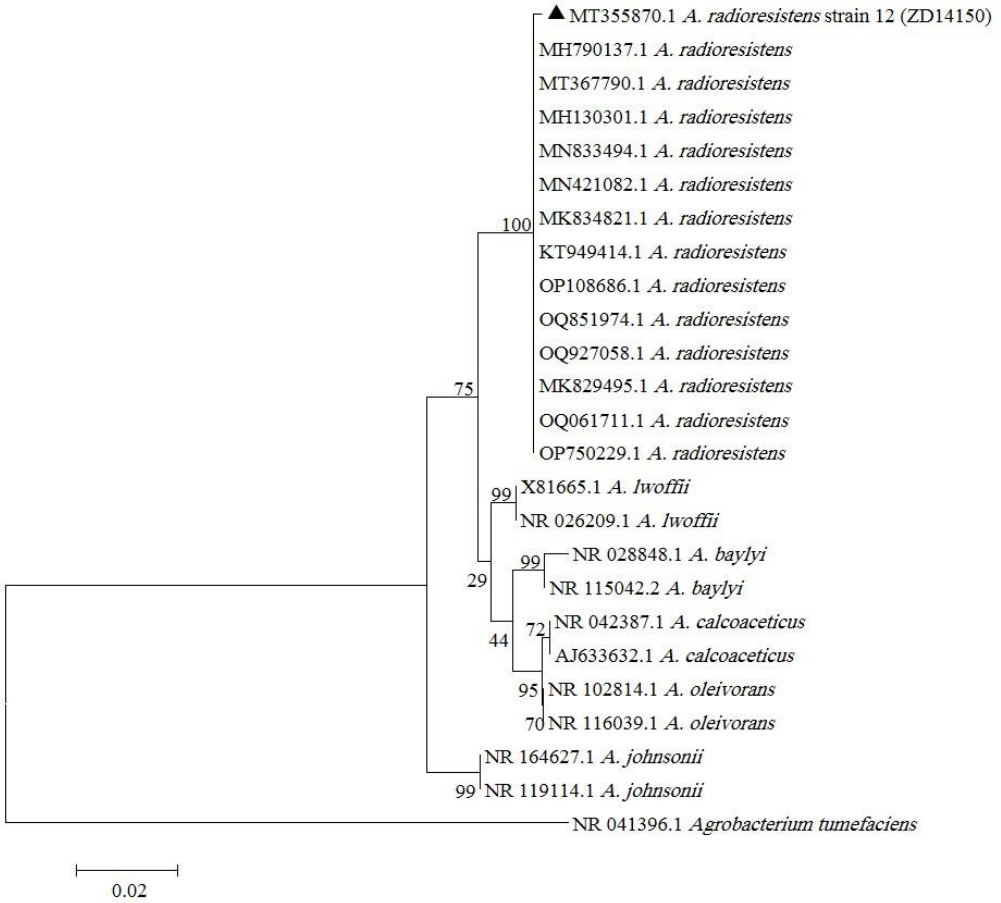

Fig. 10. Phylogenetic tree based on 16S rRNA nucleotide sequences of *Acinetobacter radioresistens* isolate (▲) and other isolates registered in the Gene Bank. The evolutionary history was inferred using the Neighbour-joining method The sequence of *Agrobacterium tumefaciens* (NR 041396) served as an out group.

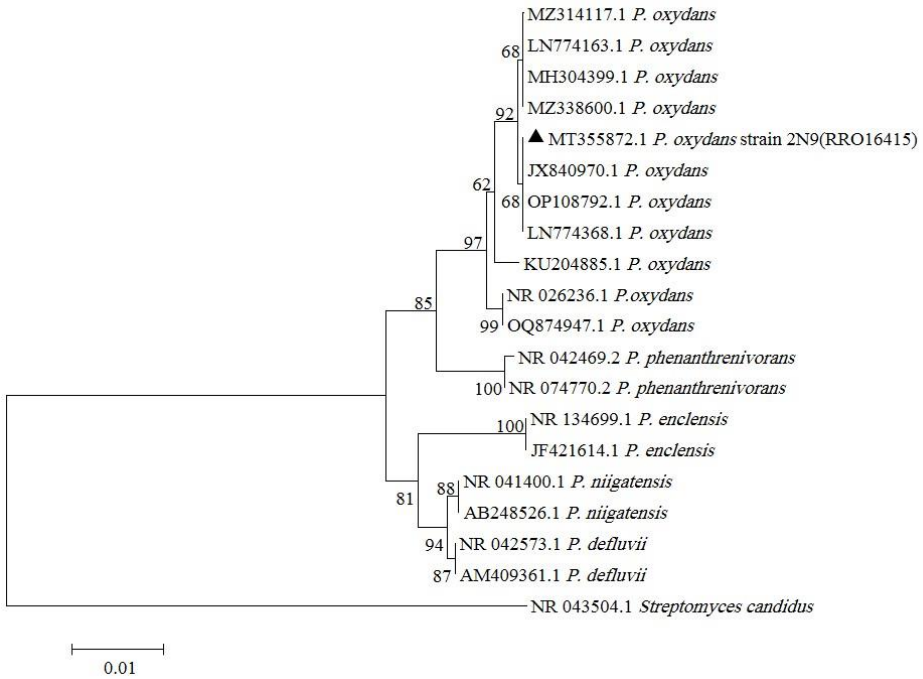

Fig. 11. Phylogenetic tree based on 16S rRNA nucleotide sequences of *Pseudarthrobacter oxydans* isolate (▲) and other isolates registered in the Gene Bank. The evolutionary history was inferred using the Neighbour-joining method The sequence of *Streptomyces candidus* (NR 043504) served as an out group.

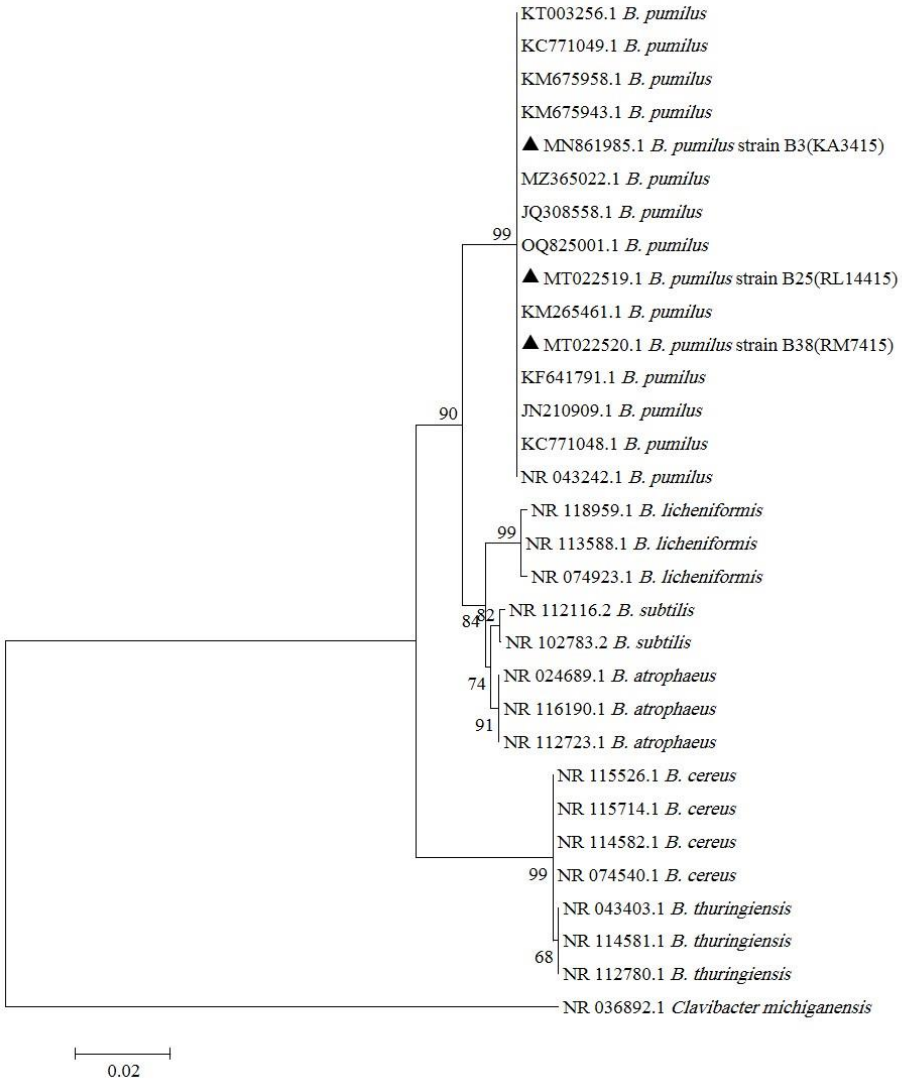

Fig. 12. Phylogenetic tree based on 16S rRNA nucleotide sequences of *Bacillus pumilus* isolates (▲) and other isolates registered in the Gene Bank. The evolutionary history was inferred using the Neighbour-joining method The sequence of *Clavibacter michiganensis* (NR 036892) served as an out group.

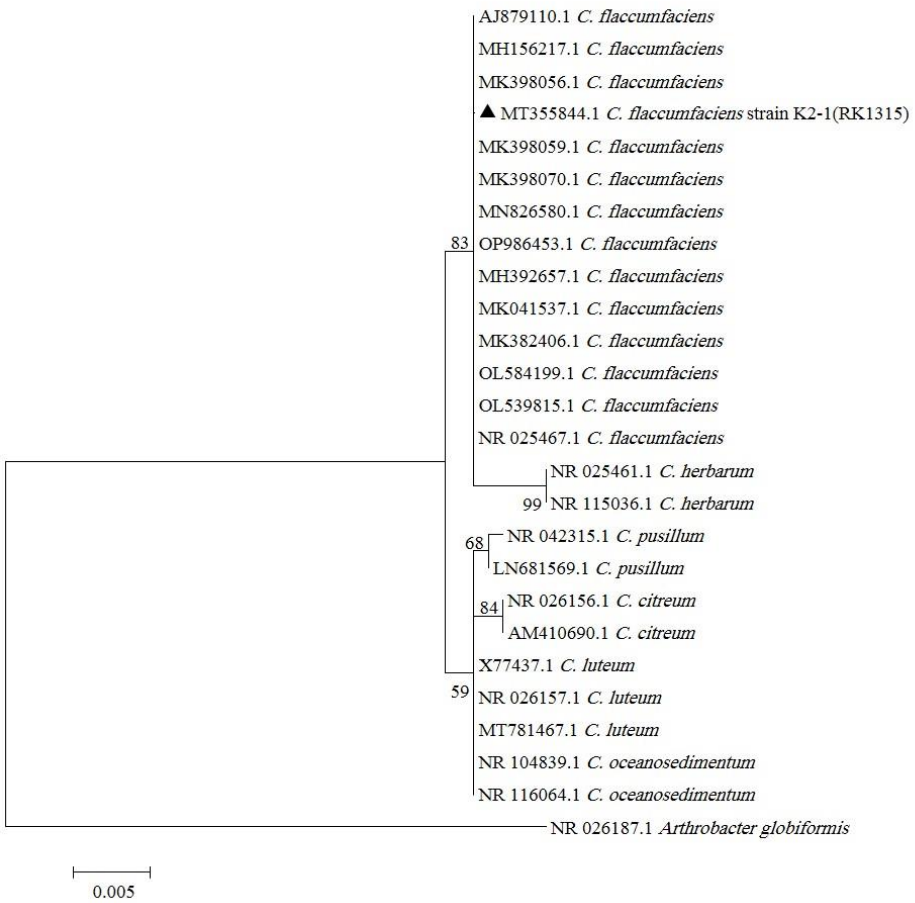

Fig. 13. Phylogenetic tree based on 16S rRNA nucleotide sequences of *Curtobacterium flaccumfaciens* isolate (▲) and other isolates registered in the Gene Bank. The evolutionary history was inferred using the Maximum-likelihood method The sequence of *Arthrobacter globiformis* (NR 026187) served as an out group.

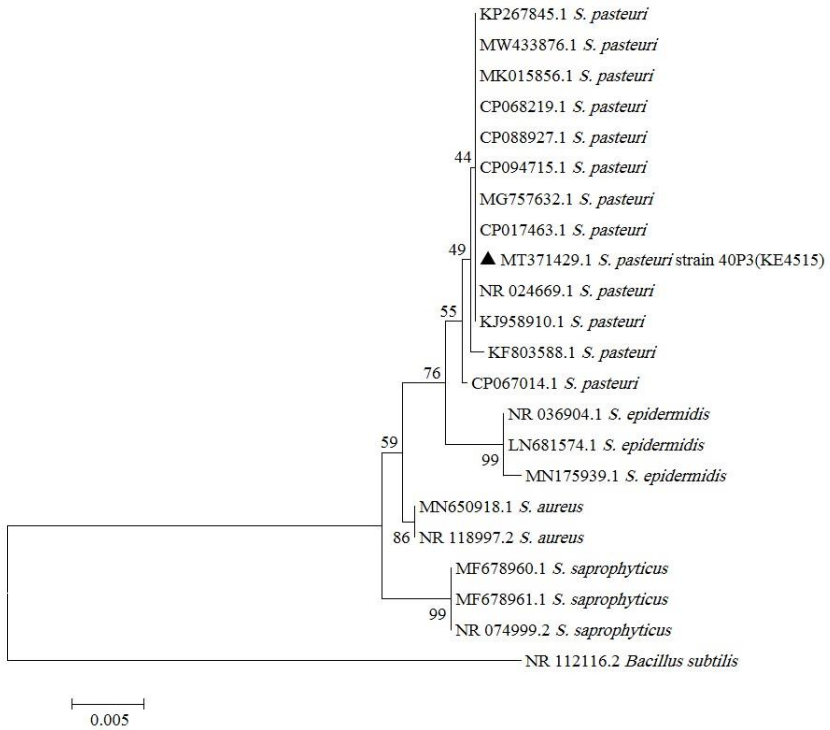

Fig.14. Phylogenetic tree based on 16S rRNA nucleotide sequences of *Staphylococcus pasteuri* isolate (▲) and other isolates registered in the Gene Bank. The evolutionary history was inferred using the Neighbour-joining method The sequence of *Bacillus subtilis* (NR 112116) served as an out group.

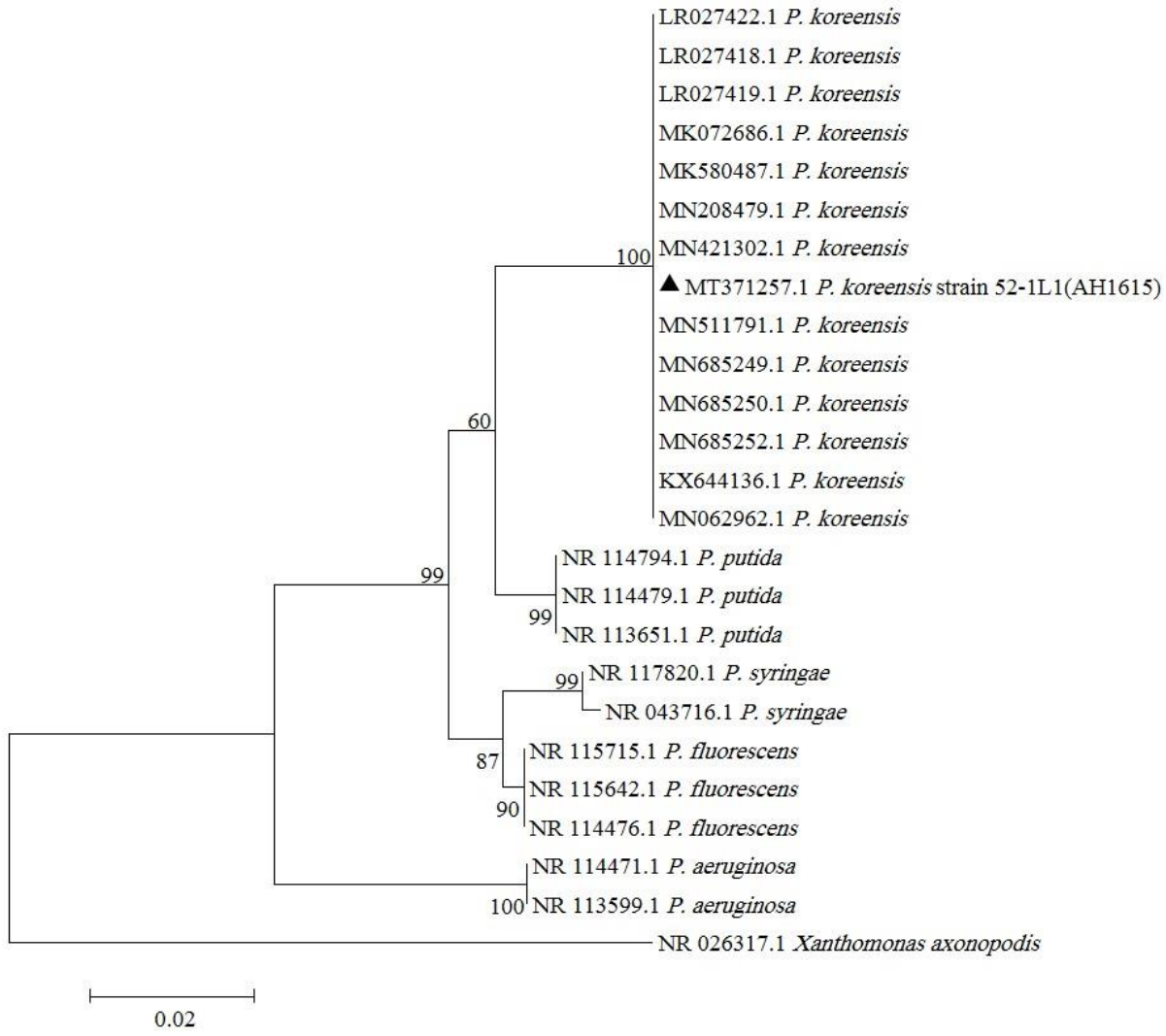

Fig. 15. Phylogenetic tree based on 16S rRNA nucleotide sequences of *Pseudomonas koreensis* isolate (▲) and other isolates registered in the Gene Bank. The evolutionary history was inferred using the Neighbour-joining method The sequence of *Xanthomonas axonopodis* (NR 026317) served as an out group.

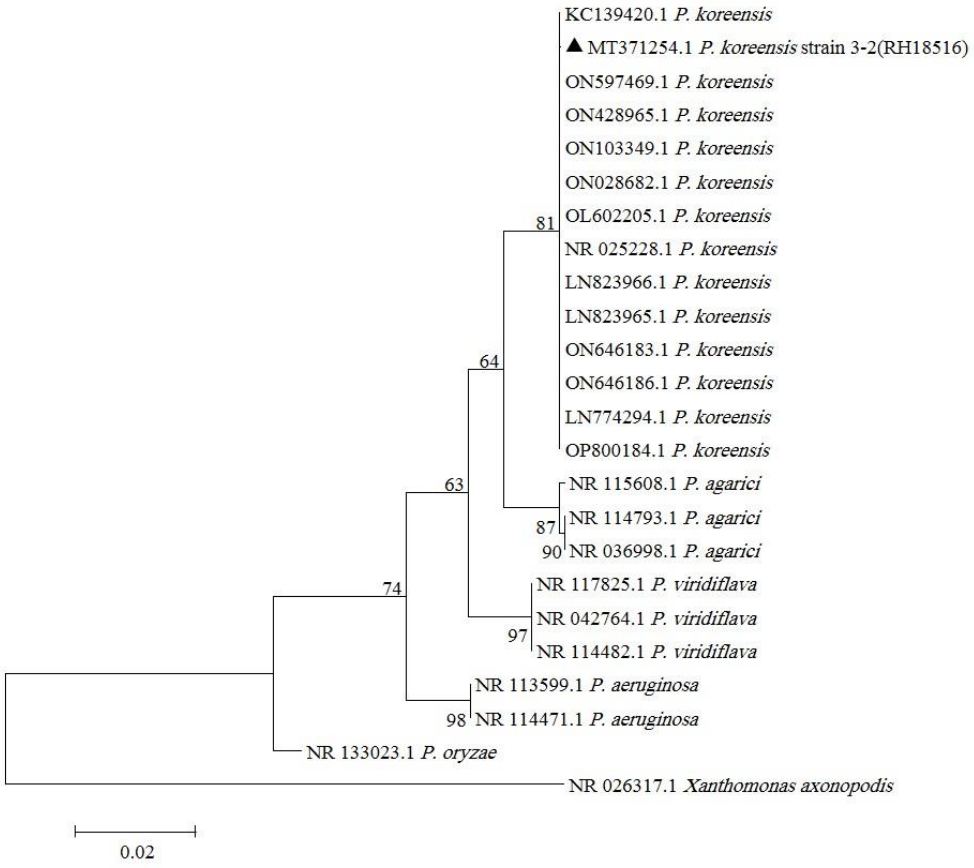

Fig. 16. Phylogenetic tree based on 16S rRNA nucleotide sequences of *Pseudomonas koreensis* isolate (▲) and other isolates registered in the Gene Bank. The evolutionary history was inferred using the Maximum-likelihood method. The sequence of *Xanthomonas axonopodis* (NR 026317) served as an out group.

Discussion

The incidence, symptoms and pathogenicity of different genera of bacteria associated with DBP were evaluated in commercial pistachio orchards in the Kerman province, Iran, as well as to distinguish the symptoms of bacterial dieback with those from other diseases such as Verticillium wilt. The field evaluations and isolations on culture media suggested that DBP may be caused by bacterial pathogens through the xylem vessels, followed by secondary infection of fungal pathogens such as *Paecilomyces* on wilted twig or branches. *P. variotii* has already been suggested as the causal agent of DBP (Alizadeh *et al*., 2000). Bacterial isolates were detected in trunk, branch and twig samples with vascular disease, while not all samples of asymptomatic wood beneath discoloration tissues were positive. Most current season shoots did not yield bacterial isolation. Longitudinal sequential cross-sections isolation from the top of twigs to primary branches downward in xylem vessels revealed the presence of fungal species with general symptoms. While bacterial isolates were detected in local symptoms and asymptomatic xylem vessels immediately adjacent to stained tissues. Bacterial isolates may have systematically invaded the xylem tissues causing water shortage due to vascular plugging. This may indicate consecutive infections occurring in the die-back of pistachio trees in Iran, where bacterial first invasion in xylem tissues is followed by secondary pathogens such as *P. variotii*. When the plants show poor growth and lack of vigor, they are naturally susceptible to diseases caused by one or more secondary infections (Agrios, 2005). Several studies have shown that plants harbor a large number of bacteria that are able to colonize, spread and move in the intercellular spaces and inside vascular elements in almost all plant species (Bacon

and Hinton, 2006; Di Fiori and Del Gallo, 1995; Lodewyckx *et al*., 2002; Ulrich *et al*., 2008).

Facelli *et al*., (2009) isolated *Xanthomonas translucens* from all parts of disease trees except the roots and new shoots causing tylosis in xylem vessels. The same authors mentioned that the staining pattern of dieback caused by bacteria may be distinguishable from other diseases such as Verticillium wilt.

Most of these bacterial genera have been reported for the first time in the current study on diseased trees in Iran which are suffering from dieback. The high abundance of *Bacillus* isolates may be due to their highly resistant to extreme environmental stresses in pistachio orchards such as heat, salinity and drought (Kazerooni *et al*., 2021). Although different strains may show different behavior depending on environmental conditions and the genetic background of the host plant.

One of the most important isolates was *C. flaccumfaciens* (RK1315), which had a high pathogenicity and vascular discoloration. Several strains of *C. flaccumfaciens* are known to act as pathogens on horticultural and ornamental plants (Araujo *et al*., 2002; Bell *et al*., 1995; Vidaver, 1982).

The ability of bacterial isolates to produce dieback has been documented on some plant species. Several studies have shown the role of *Acinetobacter*, *Staphylococcus*, *Enterobacter*, *Pseudomonas*, *Bacillus*, *Enterobacter* and *Microbacterium* in the development of dieback disease (Dunleavy, 1989; Jackson, 2009; Khan *et al*., 2014; Nishimura *et al*., 1987; Takahashi *et al*., 1997; Valdez *et al*., 2013). When pistachio dieback was observed in Australian pistachio orchards in 1992 (Edwards and Taylor 1998), *Xanthomonas translucens* isolates were identified as the causal agents, but no pathovar detected (Facelli *et al*. 2002 and 2005). Later on, molecular characterizations revealed a new pathovar pathogenic to pistachio named *Xanthomonas translucens* pv. *pistaciae* pv. nov (Giblot-Ducray *et al*. 2009).

Seasonal variations were observed in the isolation of bacteria in the xylem vascular tissue of pistachios. All samples taken from October until July were positive for the presence of bacterial isolates while during July and August, no bacterial strains could be isolated. It has been shown that the seasonal population structure changes might be caused by climatic conditions, such as temperature and relative humidity as well as the growth stages of host plants (Baldan *et al*., 2014; Bulgari *et al*. ,2014; Jansson and Douglas 2007; Mocali *et al*., 2003). Mocali *et al*., (2003) determined the fluctuations of bacterial communities by different parameters and reported strong fluctuations related to seasonal temperature variations, plant organs and the presence/absence of vascular diseases. McClean and Kluepfel (2010) reported that *Brenneria rubrifaciens* can persist in the vascular tissue of trees until a change in environmental conditions occurs, resulting in the emergence of virulent bacteria and disease development. Failure to isolate bacterial isolates in warm months may indicate their sensitivity to changing plant tissue compounds, environmental conditions and decrease in their density in vessels. Mohammadi *et al*., (2005) reported it may not be possible to isolate *Verticillium dahliae* from aerial parts of pistachio trees during the warm months of the year.

Inoculations of bacterial isolates showed that the vascular discolorations in weak trees are much more than those in healthy ones (data not shown). The accumulation of plant compounds such as polysaccharides, sugar alcohols, organic acids and the release of certain compounds via plant roots such as proteins, carbon compounds, and amino acids are also key factors in enhancing or diminishing colonization of vascular tissues of plant species (Compant *et al*., 2005; Li *et al*., 2004; Miché *et al*., 2006; Renaut *et al*., 2005; Shah 2009; Behzadi Rad *et al*., 2021).

In the present work, the capability of bacterial isolates to produce DBP under laboratory and field conditions on detached and attached branches has been demonstrated. Successful control strategies to

manage DBP in pistachio orchards must consider the role of these bacterial agents. In infected orchards, proper pruning and sanitary measures are key factors for management. Infected branches or twigs should be cut 6-10 centimeters beyond vascular discolorations to eliminate bacterial infection. Applications of copper compounds or Bordeaux mixture as a preventative measure, as well as after pruning, are helpful in reducing the risks of bacterial infections. This study is the first comprehensive investigation of bacterial isolates involved in pistachio dieback in Iran. Further research is required to develop management strategies to reduce the impact of disease in infected orchards, which is now a priority for Iranian pistachio growers.

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Conflicts of interest

All the authors declared that this is no conflict of interest in the study.

Data availability statements

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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