Bacterial Species as Causative Agents Involved in Pistachios Dieback in Iran

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Abstract

Die-back of pistachio (DBP) is a major disease affecting pistachio trees in Iran and has become a serious problem in Kerman province in recent years. In the current study, the role of bacteria as causal agents of DBP was investigated 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 produce disease and vascular colonization was studied through different inoculation methods. Identification of isolates was carried out using biochemical and physiological assays, amplification of 16SrDNA region combined with partial analyses of *gyrA* gene. A total of 281 bacterial isolates were obtained from infected trees from which 148 induced the hypersensitivity reaction on tobacco leaves. From those, 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 extend 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, Arthrobacter oxydans* and *Pseudomonas koreensis* were identified to be involved in die-back of pistachio trees. The work presented here shows that a range of bacterial genera and species may be involved with the DBP, which may be used to improve the urgently needed management of the disease.

Introduction

Die-back of pistachio (DBP) trees (*Pistacia vera*) is one of the most severe diseases, causing remarkable crop damage annually. In Iran, DBP was first observed in Kerman province (Aminaee and Ershad 1987). *Paecilomyces variotii* was reported to be associated with DBP (Aminaee and Ershad 1987). The symptoms of DBP have been observed in at least 85% of pistachio producing areas of Iran (Heidarian et al., 2018). Aerial parts of trees such as canopy and trunk and their respective components will be affected by pathogenic infections, although to different degrees. Generally, infected trees show slow growing canopy and fail to undergo reproductive growth. In severe infections, the annual growth of shoots is also reduced. The initial symptoms of DBP appear in the form of small areas of brown to black discoloration on the surface of bark in infected twigs and branches. The symptoms starting from the point of infection quickly spread in all directions in twigs and branches, longitudinally within vessels and piths and radially within parenchyma. The disease can easily be recognized due to the distinguished discolorations between the infected and healthy tissues. The color of infected bark and wood tissues changes to dark brown and black. On the surface of the bark, the margins of infected areas are distinguishable from the healthy bark based on color changes and sometimes morphological changes such as flattening. In general, DBP progresses from the top of the tree downwards (Alizadeh et al. 2000; Aminaee and Ershad 1987). Infection is perennial, and in severely infected trees, DBP will infect and kill branches over the course of some years.

The frequency of infected trees in affected orchards varied between 0 and 100% (Heidarian et al. 2018). Current reports show high prevalence of disease in the most productive pistachio producing areas of Iran– with the highest risk in Kerman province (Alizadeh et al. 2000; Ghelichi et al. 2012; Heidarian et al. 2018). In recent years, DBP has become a major concern because of the drastically decreasing pistachio production especially under poor orchard management. Several studies have reported on the role of biotic factors related to the occurrence of this disease. In Greece, *Eutypa lata* was isolated from infected pistachio branches and subsequently confirmed as a new host. *Botryosphaeria dothidea* has been demonstrated 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* from infected trees as the causal agent of DBP (Alizadeh et al. 2000). *Neoscytalidium dimidiatum* has been isolated from root and stem of pistachio trees as causal agent of pistachio die-back in Turkey (Kurt et al. 2019). In recent years, bacterial agents have been reported to be involved in DBP of pistachio disorders. Bacterial die-back of pistachio has been associated with *Xanthomonas* (Edwards and Taylor 1998). Later the causal agent has been identified as *Xanthomonas translucens* (Facelli et al. 2002; 2005). In subsequent studies, the diversity among *Xanthomonas* isolated from pistachio was investigated 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 different pathogenesis scenarios require more studies. Because of the prevailing severity of DBP in pistachio orchards in Kerman province and due to the complexity of the disease and the interference of other agents with its occurrence and severity, the current study focused only on bacterial agents associated with the disease. The intention was to screen for novel, previously non-described bacterial pathogens involved in pistachio dieback in Kerman province, Iran.

Material and Methods

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 by 38, 10, 14, 6, 3 and 6 orchards, respectively, located in Kerman province during January 2015 to December 2016. In each orchard a representative sample from different trees with clear DBP symptoms were 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 NB (nutrient broth) 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 ~ 108 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*, the most hosts susceptible to die-back of pistachio.

Lab

The ability of 148 selected bacterial isolates to produce disease was singly assessed through two inoculation methods under laboratory conditions. In the first method, two-year old, healthy twigs (30 cm length) were collected from mature trees (*P. vera* cv. *Fandoghi*). 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, then removed under sterile conditions. 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 evaluations of the isolates in both methods were carried out 20 days after inoculations were performed. 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, including: 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 field conditions. 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 conducted 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).

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

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.

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. 2a-c).

In infected orchards, all aerial parts of trees such as canopy and trunk and their components were affected by pathogenic infections 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 (Fig. 1). The initial symptoms appeared in twigs or branches, but eventually spread downward to the stems and the trunk of the tree.

Figure 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; c and d, dieback of pistachio branches b, reduced annual shoot growth with about 25 years old (*Pistacia vera* cv. *Kaleh-Ghouchi*) at the end of Autumn

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 (Fig. 2d-e). Disease progression was easily 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. 2f-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 longitudinal sections showed that in upward tissues the colonization covered all vascular tissues and in downward sections the discoloration areas were linear (Fig. 3).

Figure 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.

Figure 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 types of observed symptoms as changes in the color of the infected tissue under the bark of the branches (internal symptoms), which were 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 (Fig. 4).

Figure 4 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.

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 *Arthrobacter* 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 develop and produce 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 (Fig. 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 (Fig. 6). Although staining of wood was observed in control twigs closed to injection point, but no bacteria were isolated.

Figure 5 Vascular discolorations (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.

Figure 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 tree. 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.

Figure 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 in (1 cm, thick) inoculated twigs represent longitudinal development of discoloration upward to downward of secondary braches (*Pistacia vera* cv. *Fandoghi*).

Severity of disease symptoms as described in Fig. 8 from the two inoculation methods two and 12 months after inoculations differed statistically (Table 2). Progress was clearly visible on the pistachio trees one week 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 (Figs. 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

Source	Type III Sum of Squares	df	Mean Square	F	P value
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			

Table 2 Analysis of variance for inoculations of two-year old pistachio twigs with selected

bacterial isolates under field conditions

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), *Arthrobacter 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.

Figure 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.

Figure 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

Figure 10 PCR amplification of *gyrA* in *Bacillus pumilus* isolates 1 (KA1415), 2 (KA3415), 3 (RL14415), 4 (RM7415) using species-specific primers *gyrA*-42f and *gyrA*-1066r.

M: DNA ladder (1-kb), The red rectangle shows 1000 bp; C: Negative control (without DNA).

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	*Arthrobacter 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

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.

Strain code	Accession numbers 16S rDNA	Genus/Species	Date isolated	Type of [a] symptoms	County of origin
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.

Discussion

The incidence, symptoms and pathogenicity of different genera of bacterial associated with DBP was evaluated in commercial pistachio orchards of Kerman province, Iran, as well as to distinguish the symptoms of bacterial dieback with those of other diseases such as Verticillium wilt. The field evaluation and isolation on culture media suggested that the DBP may be caused by bacterial pathogens through xylem vessels system and followed by secondary infection of fungal pathogens such as *Paecilomyces* on wilted twig or branches. *P. variotii* has already been suggested as causal agent of DBP (Alizadeh et al. 2000). Bacterial isolates were detected in trunk, branch and twig samples with vascular disease, while in asymptomatic wood beneath discoloration tissues not all samples were positive. Most current season shoots were not yield bacterial isolation. Longitudinal sequential cross-sections isolation from top of the twigs to primary branches downward in xylem vessels revealed the presence of fungal species named 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 occur 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 exhibit 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 which are able to colonize, spread and move in the intercellular spaces and inside vascular elements in almost all plant species (Bacon and Hinton 2007; 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 pistachio 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 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, any bacterial strains could not be isolated. It has been shown that the seasonal population structure changes which might be caused by climatic conditions, such as temperature and relative humidity as well as 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 which is related to the seasonal temperature variations, plant organs and 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 decrement their density in vessels. Mohammadi et al. (2005) reported it may not be possible to isolate *Verticillium dahliae* from aerial parts of pistachio tress during warm months of year.

Inoculations of bacterial isolates showed that the vascular discolorations in weak trees is much more than those 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).

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 the bacterial infection. Applications of copper compounds or Bordeaux mixture as a preventative measure as well as after pruning are helpful to reduce the risks of bacterial infections. This study is the first comprehensive investigations 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.

Declarations

Acknowledgements

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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 analysed during the current study are available from the corresponding author on reasonable request.

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Figures

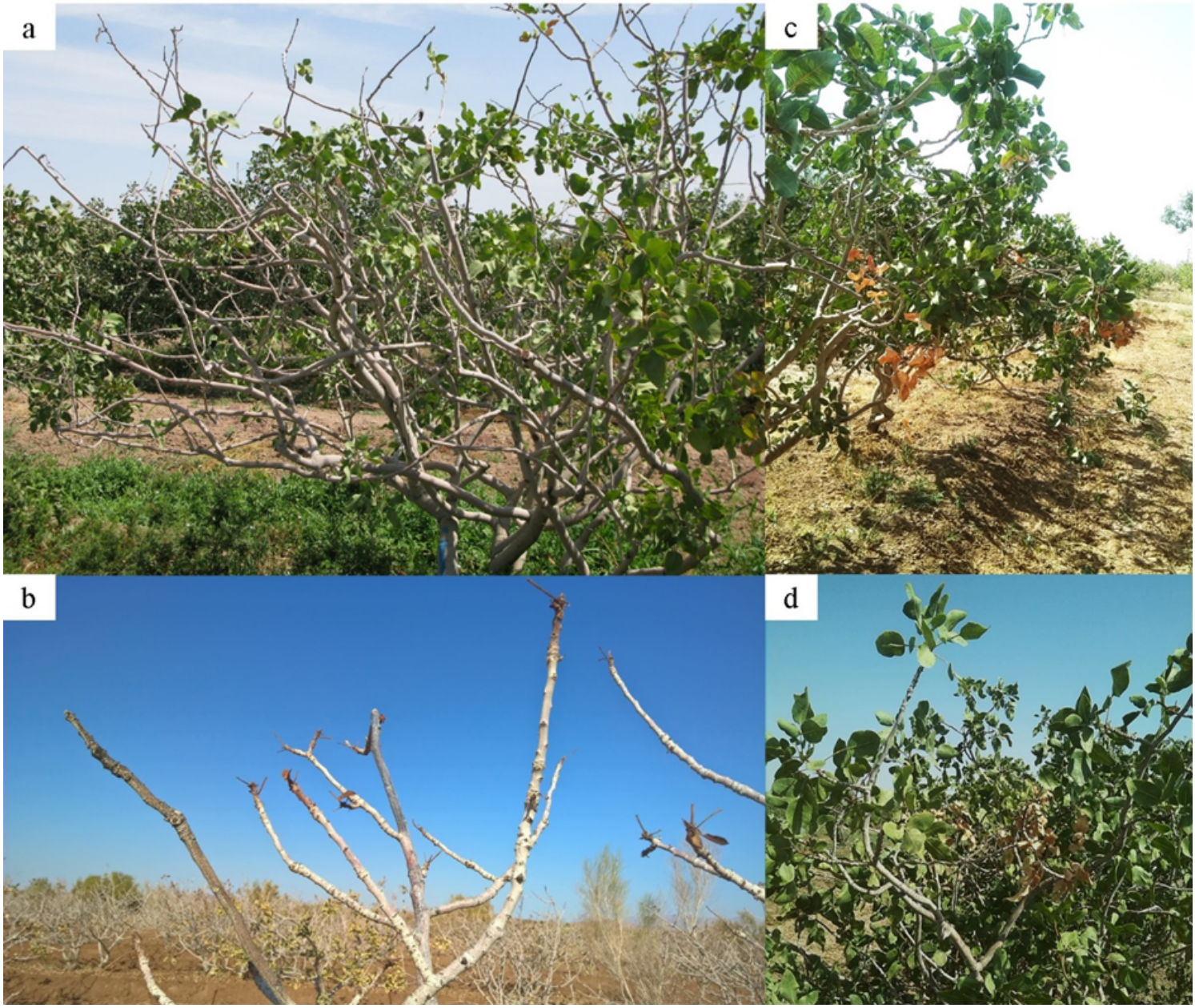

Figure 1

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; c and d, dieback of pistachio branches b, reduced annual shoot growth with about 25 years old (*Pistacia vera* cv. *Kaleh-Ghouchi*) at the end of Autumn

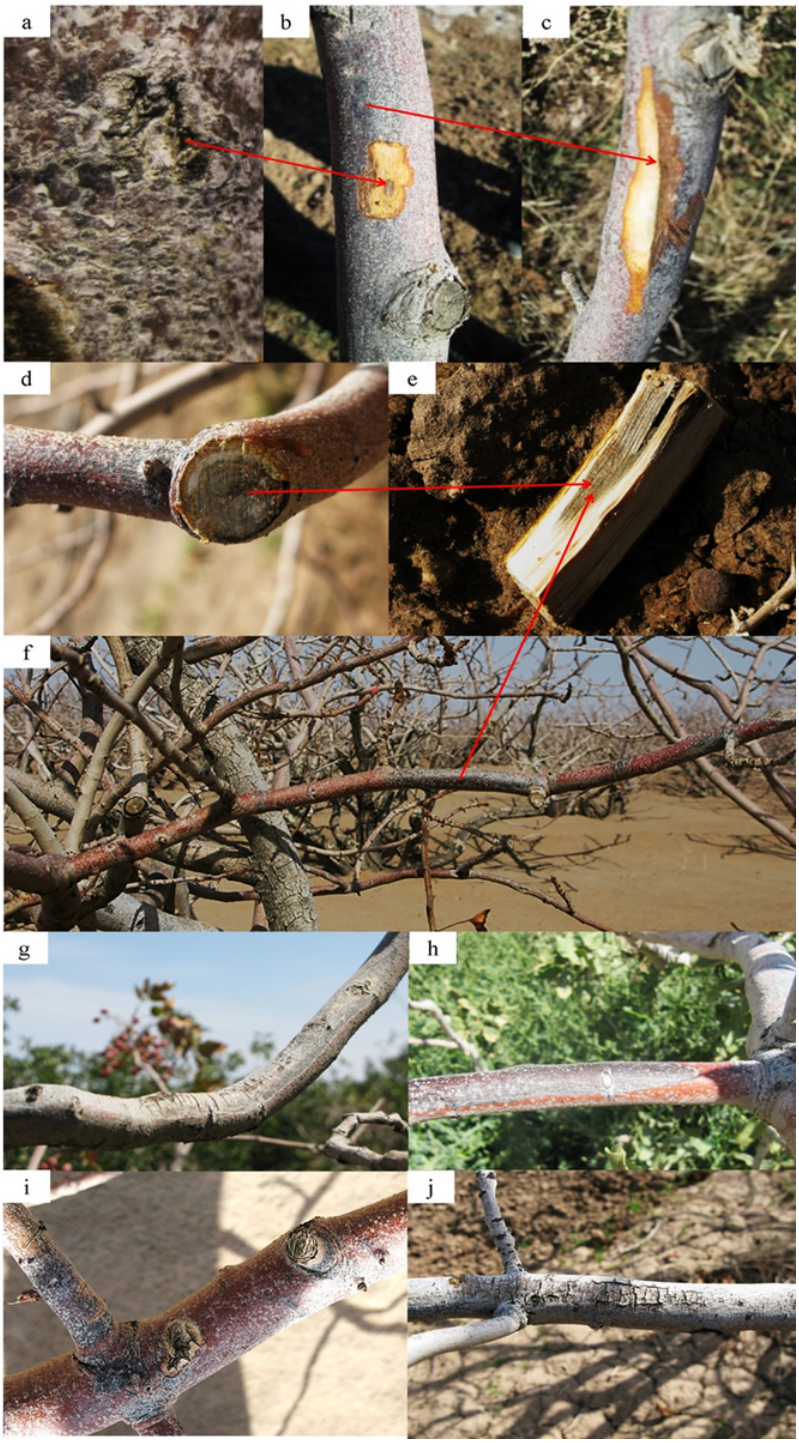

Figure 2

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 betweeninfected and healthy tissues due to the distinguished discolorations; f-j, visible symptoms on surface of bark.

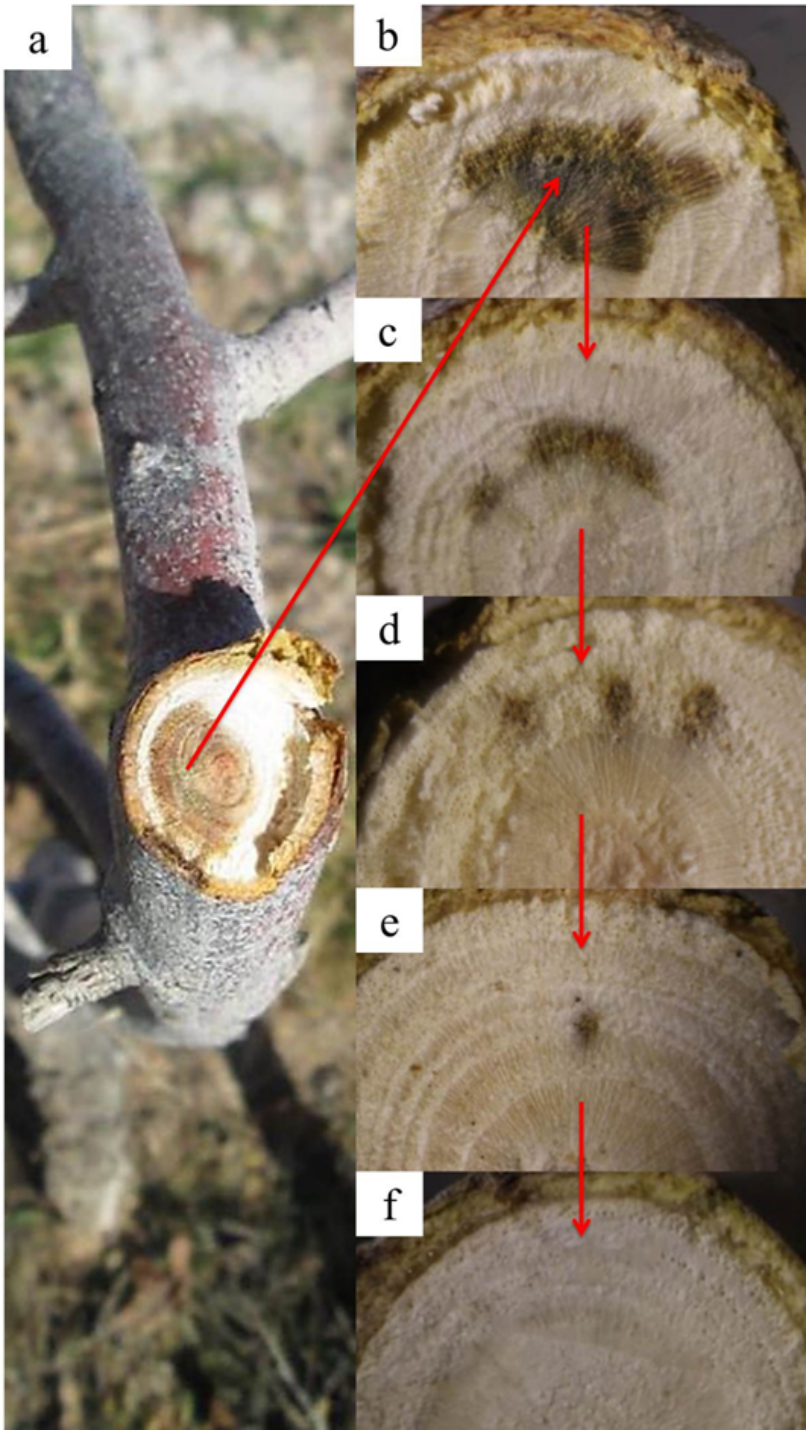

Figure 3

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.

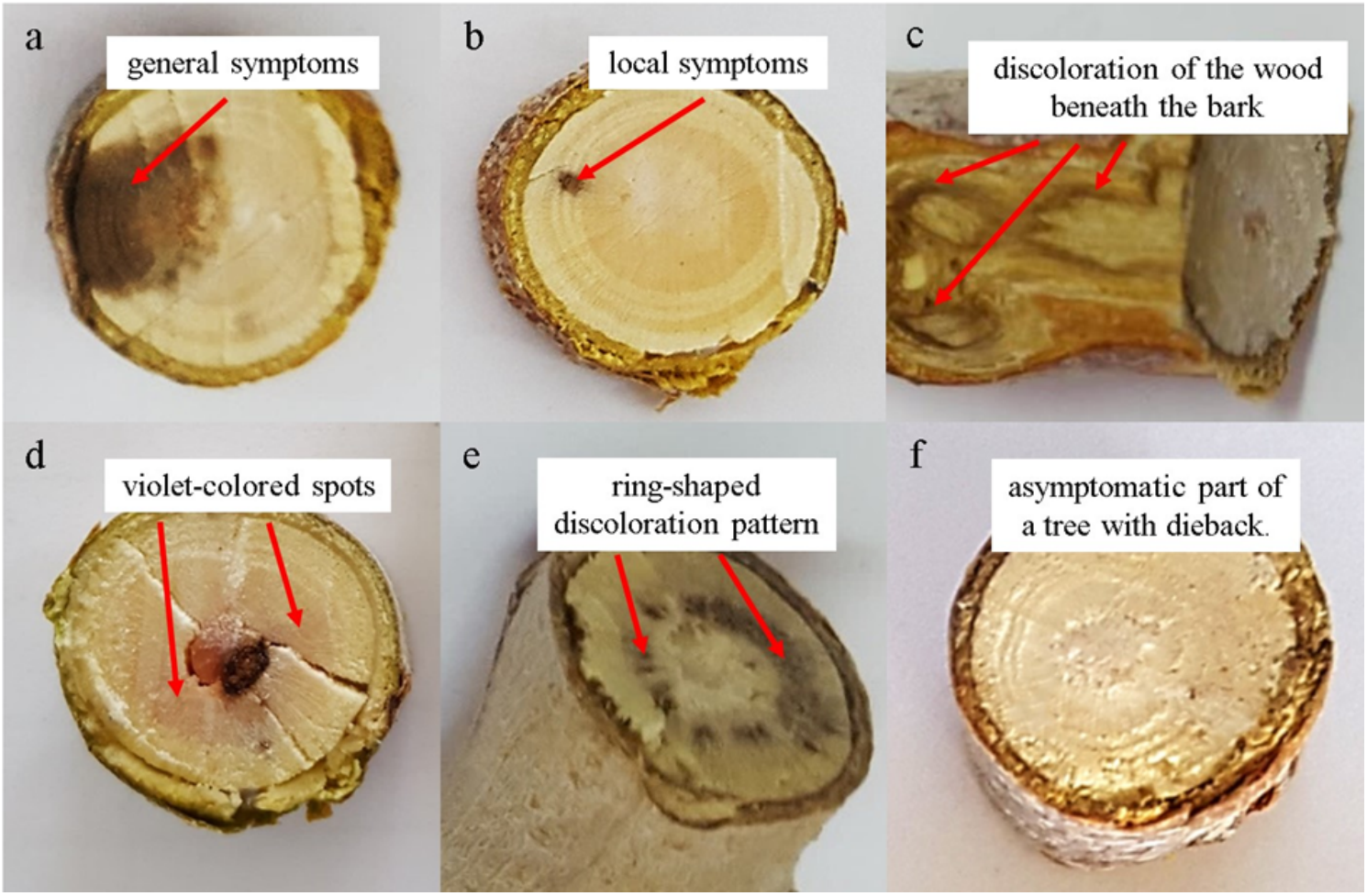

Figure 4

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.

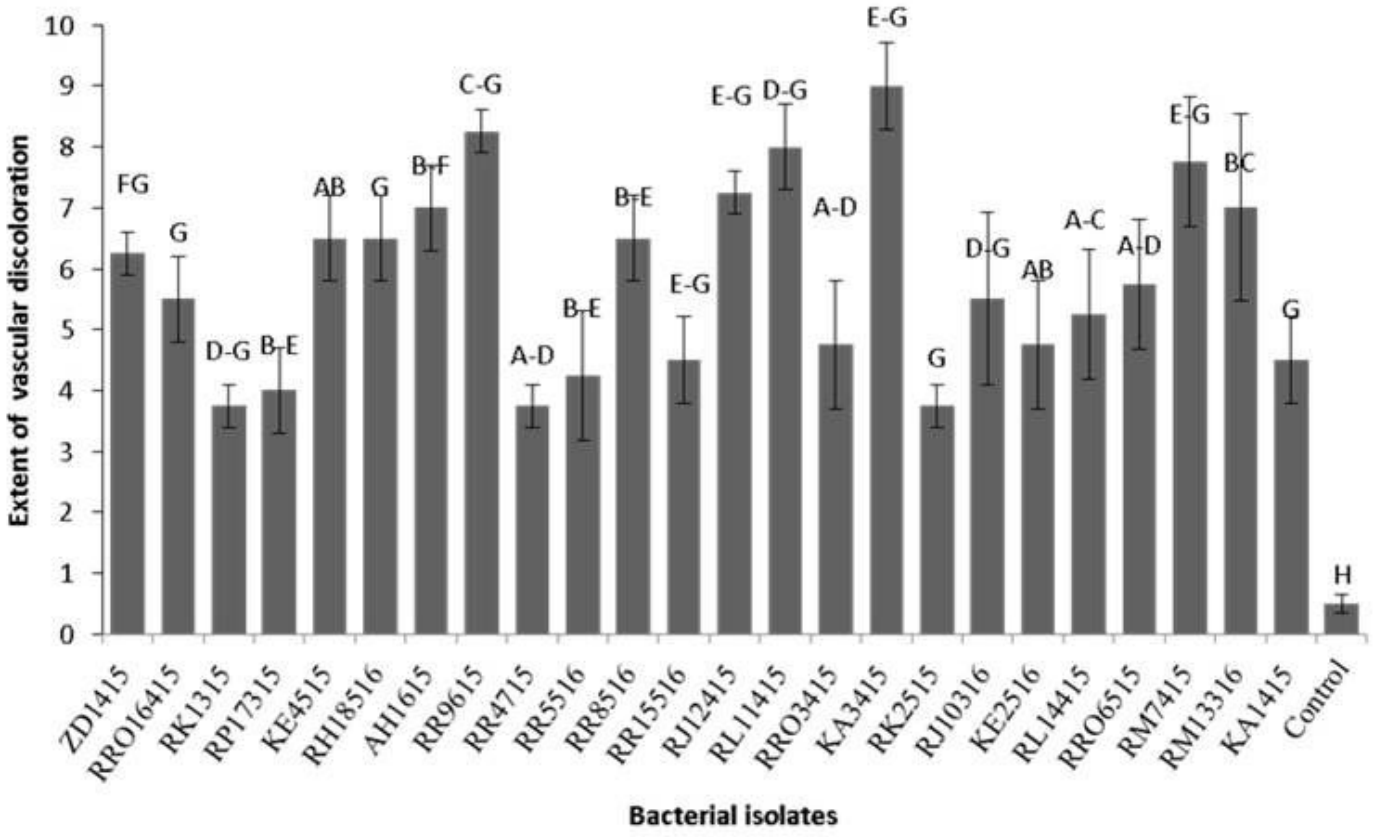

Figure 5

Vascular discolorations (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.

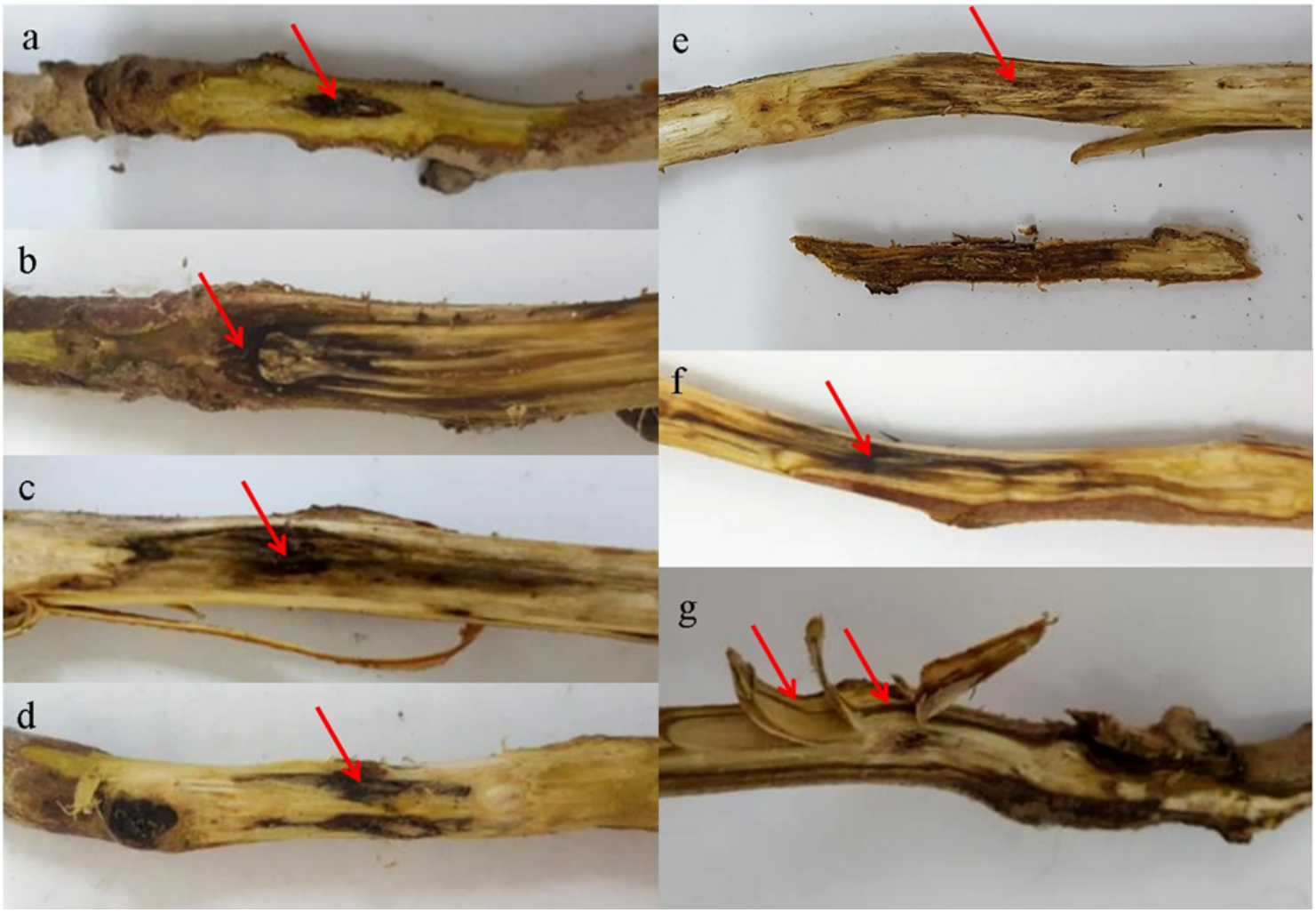

Figure 6

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.

Figure 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 in (1 cm, thick) inoculated twigs represent longitudinal development of discoloration upward to downward of secondary braches (*Pistacia vera* cv. *Fandoghi*). 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.

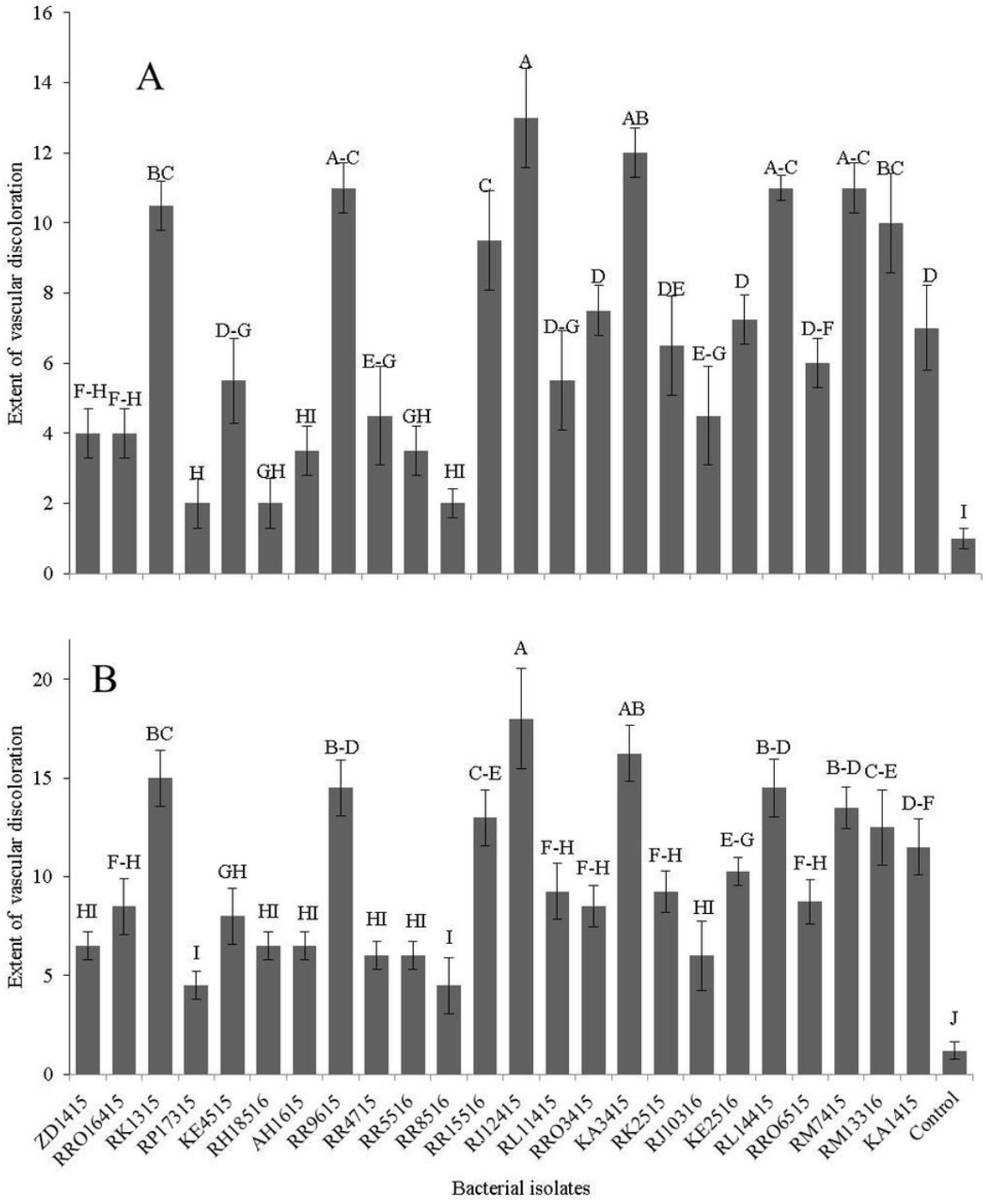

Figure 8

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.

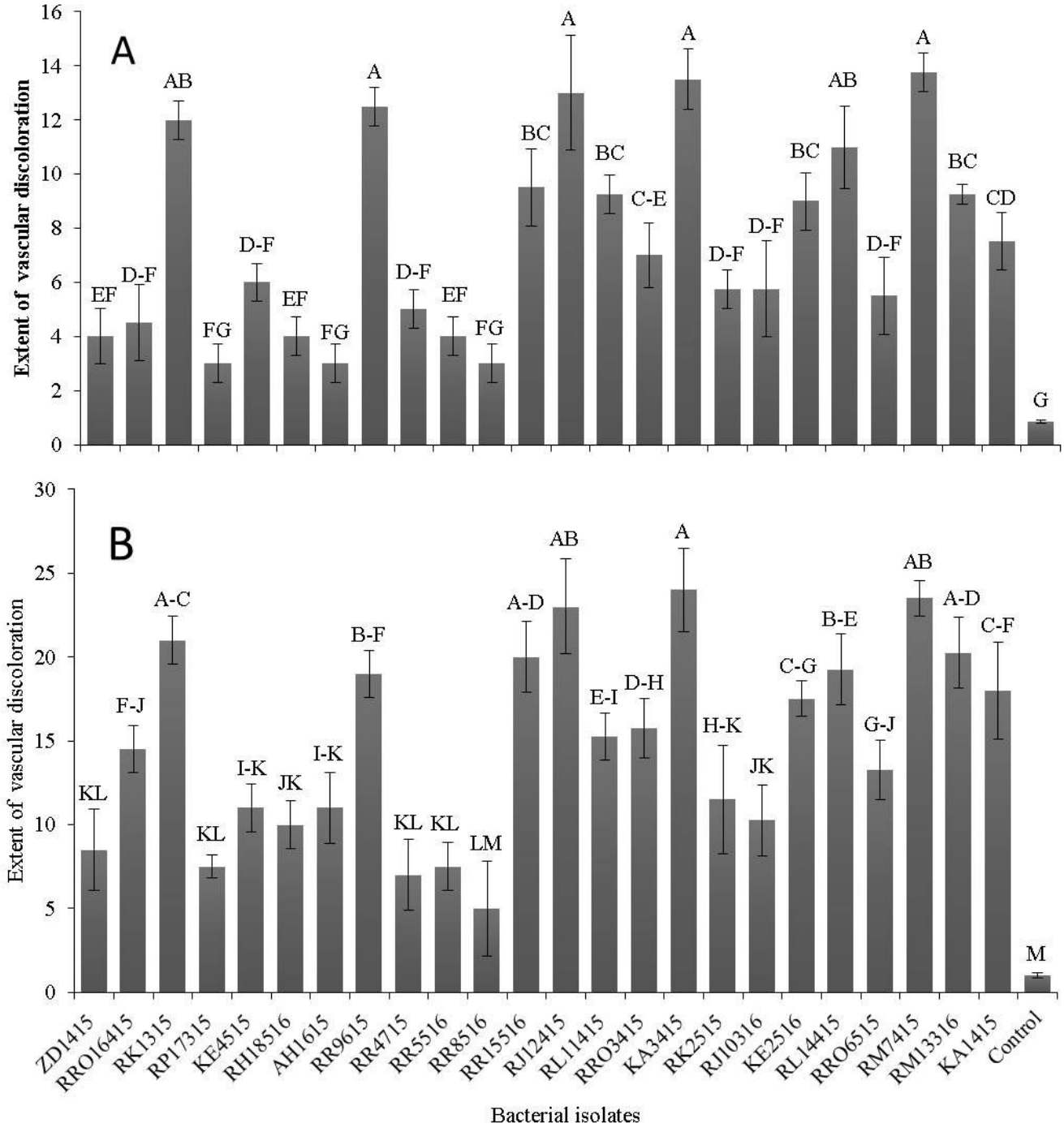

Figure 9

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

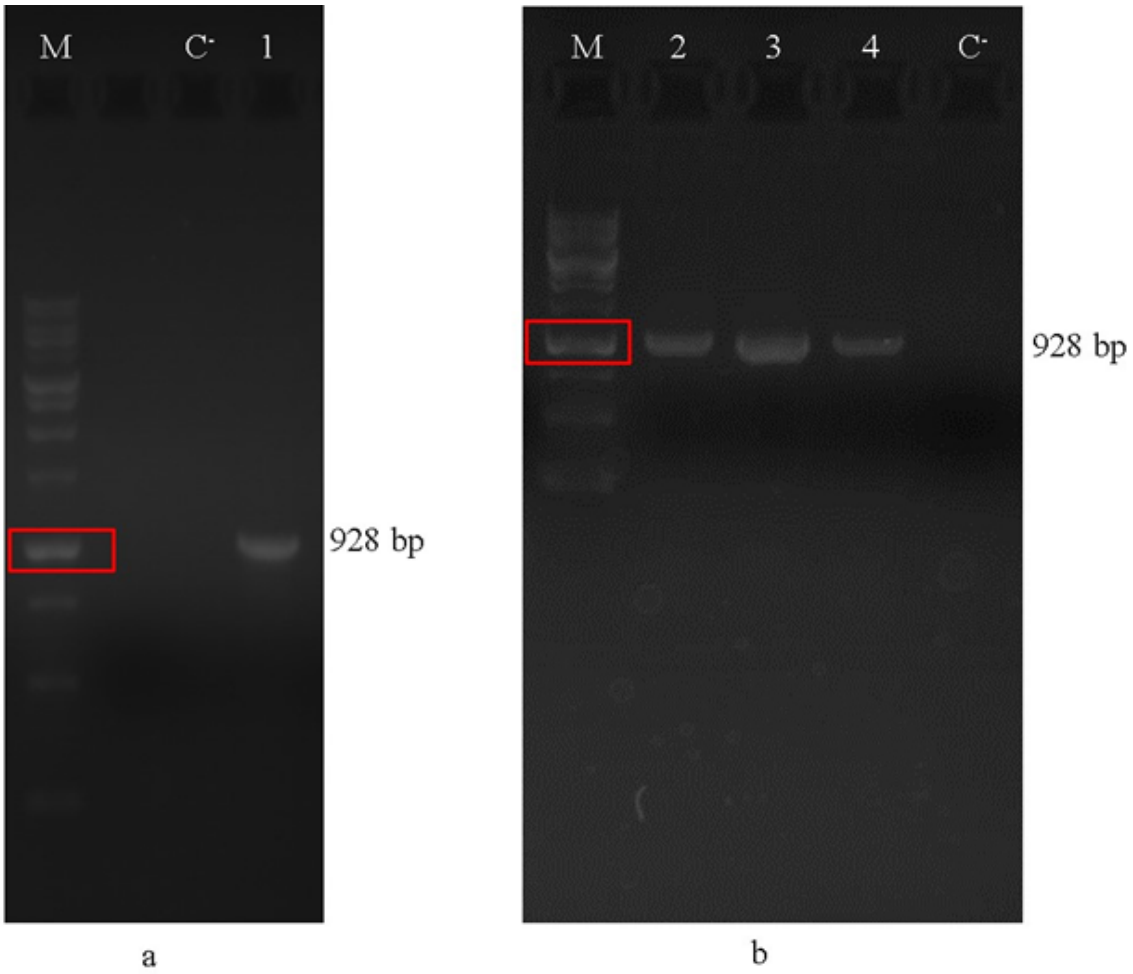

Figure 10

PCR amplification of *gyrA* in *Bacillus pumilus* isolates 1 (KA1415), 2 (KA3415), 3 (RL14415), 4 (RM7415) using species-specific primers *gyrA*-42f and *gyrA*-1066r.

M: DNA ladder (1-kb), The red rectangle shows 1000 bp**;** C: Negative control (without DNA).