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# Effects of Bacterial Strains to Inhibit Growth of *Phytophthora pistaciae* under Different Electrical Conductivities

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A B S T R A C T

Root and crown rot (gummosis) is known as the most destructive disease affecting pistachio in Iran. The efficiency of bacterial strains to reduce the growth rate of *Phytophthora pistaciae* was studied under different electrical conductivities (EC, 0, 2, 4, 8, 12 ds/m). Soil and rhizosphere samples were collected from pistachio growing regions in Kerman province, Iran, during 2011 - 2012. Overall, the strains of bacteria were presented in all sampling areas in both infected and uninfected orchards. Out of 400 bacterial isolates, 63% and 37% were collected from soil and rhizosphere samples, respectively. Among 400 bacterial isolates, 19 exhibited the highest ability to reduce the growth of *P. pistaciae* in dual culture, volatile and non-volatile compounds, though by different degrees. The degrees of inhibitory activities against mycelial growth of *P. pistacia*e by *Pseudomonas fluorescens* strains ranged from 40 to 97.5%, 8 to 97.5% and 7.5 to 90% in dual culture, non-volatile and volatile assays, respectively. The *Bacillus subtilis* strains reduced the growth of *P. pistacia*e by 22-92.5%, 17-85%, 21-92.5% in dual culture, non-volatile and volatile assays, respectively. The negative effects of ECs on the growth of *P. pistacia*e in modified CMA were observed in 8 and 12 ECs. ECs had no effect until 8 ds/m on the growth of *P. pistaciae*, while the mycelial growth decreased by ECs higher than 8 ds/m. No mycelial growth was observed at EC 14 ds/m. There were significant differences between different bacterial isolates, ECs and their interactions on the mycelial growth of *P. pistaciae*. The highest mycelial suppression belonged to isolates Nos. 123 and 112 in dual culture, volatile and non-volatile compounds test. More research is required to understand the native mechanisms involved in biological control under natural conditions in pistachio orchards

## Introduction

Both biotic and abiotic factors may affect pistachio (*Pistacia vera* L.) production (Moradi, 2015; Moradi *et al*., 2017). Crown and root rot in pistachio trees (gummosis) are caused by multiple species of the *Phytophthora* (Ershad, 1992; Banihashemi, 1994; Banihashemi and Moradi, 2004; Mirabolfathy *et al*., 2001; Fani *et al*., 2005; Mostowfizadeh-Ghalamfarsa *et al*., 2008). *Phytophthora pistaciae* and *P. drechsleri* are the most frequently found species (Mirabolfathy *et al*., 2001; Banihashemi and Moradi,

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2004). Pistachio gummosis is the most common cause of tree mortality in the orchards, leading to a remarkable crop reduction. Annual damage in terms of tree mortality is estimated to be between 2 to 11% (Moradi, 2015; Moradi *et al*., 2017). Different management strategies have been introduced including the use of resistant rootstocks, the application of cultural practice, the use of chemical pesticides as well as biological approaches (Moradi, 2015; Moradi *et al*. 2017; Fani *et al*., 2013). Mutualism of rhizobacteria or plant growth-promoting rhizobacteria (PGPR) and plants is a key factor in the biocontrol strategies for an eco-friendly management of plant diseases in many plant-microbe interactions. Disease is suppressed through induced systematic resistance (ISR) and through the production of antifungal metabolites (Vessy, 2003). *In vitro* studies have shown that different metabolites such as antibiotics, enzymes, and volatile compounds produced by *Bacillus* and *Pseudomonas* isolates are the main reasons for the phytopathogen suppression ((Haas and Défago, 2005; Lugtenberg and Kamilova, 2009). Antagonism and plant growth promotion effects of the metabolites on plant pathogens are demonstrated in natural environments (Earl *et al*., 2008). Overall, plants exposed to environmental stresses are more susceptible to root rot caused by *Phytophthora* root rot. On the other hand, environmental stress increased the severity and incidence of plant disease in most cases. Swiecki and MacDonald (1991) have shown higher root and crown rot severity in stressed tomato plants caused by *Phytophthora parasitica* than non-stressed controls. The synergistic effects were the most pronounced in young (pre-bloom) plants. Besri (1993) reported that the isolates of Phytophthora gummosis of citrus were more tolerant to soil salinity than the crop plant they attacked. The isolates produce more sporangia in saline soils than in non-saline ones (Besri 1993). It has been shown that increasing the salinity of media promotes mycelial growth of *P. citrophthora* and *P. parasitica* with an optimum of −1.44 to −3.11 bars (Benyahia, 1998) as well as the stimulation of sporangium formation of Phytophthora parasitica by salinity under *in vitro* conditions (Regragui and Lahlou, 2005). Banihashemi and Tabatabaee (2004) reported that increasing the salinity had significant effects on the growth of *P.citrophthora* and *P. nicotianae* while mycelial growth, sporangia and zoospores of *P. nicotianae* were more sensitive than *P.citrophthora.* There is not enough information on the interactions between profitable bacterial strains of pistachio rhizosphere and *Phytophthora* species in the salinity status. Therefore, the present study was conducted to evaluate the capability of bacterial isolates to inhibit *P. pistaciae* under ECs conditions.

## Materials and Methods

### *Bacterial isolates*

A total of 130 samples were collected either from soil or rhizosphere of pistachio producing areas in Kerman province during 2010-2012. Samples were taken according to a diagonal pattern in each orchard (n=24 subsamples). Samples were thoroughly homogenized in the laboratory to obtain three 100 g subsamples. Serial dilution method was used to isolate the bacteria from soil and rhizosphere on nutrient agar medium (NA, Himedia Pvt. Ltd., India). Petri-plates were incubated at 28°C for 48 hours. The suspected colonies (in terms of shape, color and size) were subcultured to a new NA plates to obtain single colonies to study antagonistic effects.

### *P. pistaciae isolate*

Originally obtained from infected trees in Rafsanjan region deposited in the culture collection at the Iranian Research Institute of Plant Protection (IRIPP), a virulent strain of *P. pistaciae* was used in all experiments. The isolate was re-cultured and maintained on either Potato Dextrose Agar (PDA, Merck, Germany) or Corn Meal Agar (CMA) (Himedia, Pvt. Ltd., India) before use.

***Screening of bacterial antagonistic***

Abilities of isolated bacteria to reduce mycelial growth of *P. pistaciae* were assessed in dual culture (Aghighi *et al*., 2004), volatile compounds (Hora and Baker, 1972) and non-volatile compounds (Berg and Ballin, 1995) assays.

***Effect of salinity on mycelial growth of P. pistacia***

The ability of selected bacterial strains to withstand two salt compounds (NaCl and $CaCl_2$) was tested in different electrical conductivities (ECs). Briefly, modified corn mail agar (MCMA) medium was prepared with different levels of NaCl and $CaCl_2$ salts in the range of 0, 2, 4, 8, 10, 12, 14 and 20 ds/m according to Richards (1954), and was measured using an electrical conductivity meter.

To evaluate the mycelium growth inhibition, a 5 mm agar plug of *P. pistaciae* was placed in the center of a petri dish plate containing the MCMA medium, and incubated at 27˚C in a dark environment. The plates were monitored every 48 hours for 7 days to measure mycelial growth and colony diameters (Aghighi *et al.* 2004).

***Effect of salinity on the antagonistic efficiency of bacterial strains***

The effect of salinity on the antagonistic efficiency of bacterial strains to reduce mycelial growth of *P. pistaciae* was assessed through dual culture, volatile and non-volatile compounds in modified CMA medium, as already described (Aghighi *et al*., 2004; Hora and Baker, 1972; Berg and Ballin, 1995).

The ability of the bacterial strains to inhibit fungal growth of *P*. pistaciae was calculated using the following formula:

$$I = (C\text{-}T) / C \times 100$$

where *I* is the inhibition percentage of mycelial growth, *C* is the mycelial growth in control plates and *T* is mycelial growth in the treatments.

***Identification of bacterial strains***

The nineteen selected bacterial strains with the highest antagonistic activity were identified based on morphological and biochemical characteristics (Shaad *et al*., 2001).

***Statistical analysis***

The experiments were carried out based on completely randomized design with three replicates. The average values of mycelial growth were separately calculated for each replication. Mean comparisons were done using Duncan's new multiple range test at 1 % probability. If needed, data was log-transferred prior to the analysis.

**Results**

Overall, bacteria strains were isolated in all sampling areas although their frequencies were different in both infected and uninfected orchards. Out of 400 bacterial isolates, 63% and 37% were collected from soil and rhizosphere samples, respectively. Among 400 bacterial isolates, 170 were able to inhibit the growth of *P. pistaciae* in screening through dual culture assays. Out of 170 isolates, 19 had shown the highest ability to reduce growth of *P. pistaciae* in dual culture, volatile and non-volatile compounds (Table 1). Bacterial strains with the highest antagonistic potential were identified based on biochemical and morphological characterizations (Table 2).

The intensity of inhibitory activities against mycelial growth of *P. pistacia*e by *P. fluorescens* ranged from 40 to 97.5%, 8 to 97.5% and 7.5 to 90% in dual culture, non-volatile and volatile assays, respectively (Table 1). The *Bacillus subtilis* strains reduced the growth of *P. pistacia*e by 22-92.5, 17-85, 21-92.5 in dual culture, non-volatile and volatile assays, respectively. Among the 19 isolates, isolates Nos. 123 and 112 showed the highest ability to reduce mycelial growth of *P. pistaciae* compared to other isolates. As shown in Table 2, the growth of *P. pistaciae* was not significantly affected by 2 and 4ECs (ds/m) compared

to the control in CMA medium either in presence or absence of bacterial isolates. On the other hand, the effects of ECs in modified CMA were significant in 8 and 12ECs (ds/m), which reduced the mycelial growth of *P. pistaciae* ranged respectively from 38 to 92.5% and 40 to 97.5% in dual culture, 31.5 to 92 and 37.5 to 97.5 in non-volatile compounds and 34 to 83.5 and 7.5 to 92.5 in volatile compounds assays. The growths of *P. pistaciae* in different ECs have shown no effects until 8 ds/m while the mycelial growth decreased in the ECs higher than 8 ds/m. No mycelial growth was observed at ECs 14 ds/m. There were significant differences between the isolates of bacteria, ECs and their interactions to reduce mycelial growth of *P. pistaciae* (Table 3). The highest mycelial reductions belonged to isolates Nos. 123 and 112 in dual culture, volatile and non-volatile compound tests.

**Table 1. The effect of bacterial strains on mycelial growth (CM) of *Phytophthora pistaciae* in different electrical conductivities by various competition tests**

| | Ec | Bacterial strains | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 123 | 32 | 28 | 112 | 146 | 151 | 23 | 153 | 154 | 143 | 150 | 29 | 79 | 149 | 6 | 78 | 157 | 40 | 156 | Control |
| Dual Culture | 0 | 0.9 | 1.9 | 2.3 | 0.9 | 3.9 | 4.0 | 3.4 | 3.7 | 1.3 | 1.3 | 1.6 | 1.3 | 4.5 | 4.3 | 6.7 | 1.7 | 5.0 | 1.9 | 4.1 | 8.6 |
| | 2 | 0.7 | 1.5 | 2.0 | 0.8 | 3.6 | 4.0 | 3.3 | 3.6 | 1.3 | 1.3 | 1.5 | 1.3 | 4.0 | 3.8 | 4.7 | 1.6 | 3.0 | 1.7 | 3.8 | 8.5 |
| | 4 | 0.7 | 1.7 | 2.0 | 0.8 | 3.4 | 3.6 | 3.3 | 3.5 | 1.2 | 1.2 | 1.3 | 1.2 | 3.7 | 3.6 | 4.4 | 1.3 | 3.3 | 1.5 | 3.7 | 8.7 |
| | 8 | 0.4 | 1.2 | 1.4 | 0.4 | 2.3 | 2.4 | 1.9 | 1.7 | 0.8 | 0.8 | 1.0 | 0.8 | 2.9 | 2.5 | 2.9 | 1.2 | 4.5 | 1.4 | 2.4 | 7.3 |
| | 12 | 0.1 | 0.5 | 2.4 | 0.1 | 1.3 | 1.4 | 1.0 | 1.0 | 0.3 | 0.3 | 0.5 | 0.3 | 1.5 | 1.3 | 1.6 | 0.5 | 2.2 | 0.7 | 1.4 | 4.0 |
| | **LSD** | **0.2** | **0.5** | **2.6** | **0.2** | **0.5** | **0.5** | **0.3** | **0.4** | **0.4** | **0.3** | **0.2** | **0.3** | **0.6** | **0.6** | **0.4** | **0.2** | **0.8** | **0.4** | **0.6** | **1.4** |
| Non-Volatile compounds | 0 | 1.3 | 2.6 | 3.8 | 1.3 | 5.8 | 5.4 | 4.8 | 5.2 | 2.8 | 2.4 | 2.7 | 7.9 | 4.5 | 5.5 | 7.1 | 2.5 | 5.5 | 2.8 | 4.9 | 8.6 |
| | 2 | 1.5 | 2.6 | 2.8 | 1.8 | 4.0 | 5.2 | 4.5 | 5.2 | 2.8 | 2.4 | 2.5 | 7.3 | 4.0 | 5.1 | 5.7 | 2.4 | 3.7 | 2.5 | 4.0 | 8.5 |
| | 4 | 1.8 | 2.7 | 3.5 | 1.6 | 4.1 | 4.9 | 4.3 | 4.9 | 2.7 | 2.4 | 2.4 | 7.0 | 3.7 | 4.8 | 5.4 | 2.4 | 3.7 | 2.4 | 4.0 | 8.7 |
| | 8 | 0.6 | 1.7 | 2.1 | 0.8 | 2.8 | 2.9 | 2.7 | 2.4 | 1.8 | 1.7 | 1.7 | 5.0 | 2.9 | 3.2 | 3.5 | 1.6 | 4.8 | 1.5 | 2.7 | 7.3 |
| | 12 | 0.1 | 0.9 | 1.1 | 0.2 | 1.5 | 1.4 | 1.2 | 1.2 | 0.7 | 0.5 | 0.6 | 2.5 | 1.5 | 1.8 | 1.8 | 0.7 | 2.4 | 0.9 | 1.5 | 4.0 |
| | **LSD** | **0.5** | **0.3** | **0.6** | **0.2** | **0.5** | **0.3** | **0.5** | **0.5** | **0.5** | **0.2** | **0.3** | **0.4** | **0.6** | **0.8** | **0.5** | **0.3** | **0.4** | **0.3** | **0.3** | **1.4** |
| Volatile com-pounds | 0 | 1.2 | 2.3 | 3.7 | 2.6 | 5.2 | 5.0 | 4.7 | 4.6 | 2.6 | 2.3 | 2.7 | 7.7 | 4.5 | 5.3 | 6.8 | 2.2 | 5.1 | 2.4 | 4.5 | 8.6 |
| | 2 | 1.2 | 2.3 | 3.7 | 2.6 | 3.5 | 4.9 | 4.0 | 5.0 | 2.5 | 2.2 | 2.3 | 7.1 | 3.8 | 4.7 | 5.4 | 2.1 | 3.2 | 2.4 | 3.8 | 8.5 |
| | 4 | 1.2 | 2.3 | 3.7 | 2.6 | 3.4 | 4.7 | 4.1 | 4.7 | 2.5 | 2.2 | 2.2 | 6.8 | 3.6 | 4.3 | 5.2 | 2.2 | 3.4 | 2.2 | 3.9 | 8.7 |
| | 8 | 1.2 | 2.3 | 3.7 | 2.6 | 2.5 | 2.8 | 2.2 | 2.1 | 1.5 | 1.5 | 1.6 | 4.8 | 2.5 | 2.8 | 3.1 | 1.4 | 4.6 | 1.4 | 2.5 | 7.3 |
| | 12 | 1.2 | 2.3 | 3.7 | 2.6 | 1.3 | 1.2 | 1.0 | 1.0 | 0.3 | 0.4 | 0.5 | 2.3 | 1.3 | 1.4 | 1.5 | 0.6 | 2.1 | 0.7 | 1.4 | 4.0 |
| | **LSD** | **0** | **0** | **0** | **0** | **0.4** | **0.2** | **0.4** | **0.5** | **0.5** | **0.3** | **0.3** | **0.4** | **0.5** | **0.8** | **0.5** | **0.3** | **0.3** | **0.5** | **0.3** | **1.4** |

**Table 2. Characterizations of bacterial strains and their efficiency on *P. pistaciae* mycelial growth reduction**

| No. | IPRC code | Bacterial species | Location | Sampling area | Orchard contamination to gummosis | Mycelial growth reduction rate (%) | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Dual culture | Non-Volatile compounds | Volatile compounds |
| 1 | 23 | *Pseudomonas fluorescens* | Tajabad (Rafsanjan) | Rhizosphere | – | 60-75 | 44-70 | 45-75 |
| 2 | 28 | *Pseudomonas fluorescens* | Esmailabad (Nough) | Rhizosphere | – | 40-81 | 56-72.5 | 7.5-57 |
| 3 | 29 | *Pseudomonas fluorescens* | Esmailabad (Nough) | Soil | + | 85-92.5 | 8-37.5 | 10-42.5 |
| 4 | 32 | *Pseudomonas fluorescens* | Tajabad (Rafsanjan) | Rhizosphere | + | 69-77.5 | 69-77.5 | 42.5-73.5 |
| 5 | 79 | *Pseudomonas fluorescens* | Khalilabad | Rhizosphere | + | 48-62.5 | 48-62.5 | 48-67.5 |
| 6 | 112 | *Pseudomonas fluorescens* | Sirjan | Rhizosphere | + | 89.5-97.5 | 79-95 | 35-70 |
| 7 | 123 | *Pseudomonas fluorescens* | Sirjan | Rhizosphere | – | 89.5-97.5 | 79-97.5 | 70-86 |
| 8 | 143 | *Pseudomonas fluorescens* | Tajabad (Rafsanjan) | Rhizosphere | + | 85-92.5 | 72-87.5 | 73-90 |
| 9 | 146 | *Pseudomonas fluorescens* | Kerman | Soil | – | 55-68 | 32.5-62.5 | 39.5-67.5 |
| 10 | 149 | *Pseudomonas fluorescens* | Kerman | Soil | – | 50-67.5 | 36-56 | 38-65 |
| 11 | 151 | *Pseudomonas fluorescens* | Kerman | Rhizosphere | + | 53-67 | 37-65 | 42-70 |
| 12 | 153 | *Pseudomonas fluorescens* | Kerman | Rhizosphere | – | 57-77 | 39-70 | 41-75 |
| 13 | 6 | *Bacillus subtilis* | Kousarriz (Rafsanjan) | Rhizospher | + | 22-60 | 17-55 | 21-62.5 |
| 14 | 40 | *Bacillus subtilis* | Anar | Rhizosphere | – | 78-83 | 67-79 | 72-82.5 |
| 15 | 78 | *Bacillus subtilis* | Khalilabad | Rhizosphere | – | 80-87.5 | 71-82.5 | 74-85 |
| 16 | 150 | *Bacillus subtilis* | Kerman | Soil | – | 81-87.5 | 69-85 | 69-87.5 |
| 17 | 154 | *Bacillus subtilis* | Kerman | Soil | + | 85-92.5 | 67-82.5 | 70-92.5 |
| 18 | 156 | *Bacillus subtilis* | Kerman | Soil | – | 52-67 | 43-63 | 48-66 |
| 19 | 157 | *Bacillus subtilis* | Kerman | Soil | – | 38-65 | 34-57 | 37-62 |

**Table 3. Analysis of variance of growth inhibition of *P. pistaciae* under *in vitro* experiments.**

| Source | Treatment | df | sum of square | Mean of Square | P |
|---|---|---|---|---|---|
| Dual culture | Bacteria | 17 | 739.47 | 38.91 | <.0001 |
| | EC | 4 | 156.87 | 39.21 | <.0001 |
| | Bacteria * EC | 76 | 85.88 | 1.13 | <.0001 |
| Non-volatile | Bacteria | 17 | 693.53 | 36.5 | <.0001 |
| | EC | 4 | 362.56 | 90.64 | <.0001 |
| | Bacteria * EC | 76 | 93.86 | 1.23 | <.0001 |
| Volatile | Bacteria | 17 | 622.85 | 32.78 | <.0001 |
| | EC | 4 | 271.03 | 67.75 | <.0001 |
| | Bacteria * EC | 76 | 138.93 | 1.828 | <.0001 |

## Discussion

One of the major factors reducing pistachio quality and quantity is the increase in the salinity of soil and water in recent years. Electrical conductivity in soil and water is about 2.0 to more than 20 dS/m, which have been showing an increasing trend in recent years because of dropping underground water table and as a consequence of water quality decrease. The current study has shown the presence of native strains of bacterial with an ability to control *Phytophthora* crown and root rot of pistachio trees under saline and non-saline conditions. The bacterial isolates are already adapted to biotic and abiotic stresses found in the orchards and may have a higher stability to control pistachio gummosis. This requires more research to understand the dynamics of bacterial isolates in soil and rhizosphere (Lee *et al*., 2015).

The effects of different ECs levels were compared on the mycelial growth of *P. pistaciae* in a modified medium. No effect was observed on the mycelial growth of *P. pistaciae* in 4 and 8 ECs while a salinity of higher than 8 ds/m decreased the mycelial growth to different degrees. Here, it is shown that the bacterial isolates could inhibit mycelia growth of *P. pistaciae* either in the presence or absence of salt stresses, which was more pronounced in higher concentrations of salt in the media. Several studies have shown an increase of mycelial growth and spore germination by chloride salts in low concentrations, while high concentrations inhibit the growth and spore germination of many plant pathogenic fungi compared to the control – although by different degrees (Benyahia, 1998; Biggs *et al*., 1997; Regragui and Lahlou, 2005). The effects of salts in reducing growth of fungal species may be due to toxicity in high concentrations affecting the osmotic balance or pressure in fungal cells, which inhibit biological activity (Arras *et al*., 1998). On the other hand, in low concentrations, the effect of salinity to stimulate the growth of fungal species is under the effect of specific ions (Wisniewski *et al*., 1995). In the present study, there were significant differences between isolated bacteria to reduce the growth of *P. pistachio* with the highest effects, which were observed in *Pseudomonas fluorescens* isolates Nos. 112 and 123 in all *in vitro* assays. Raaijmakers *et al*., (2010) have shown that the production of volatile and non-volatile compounds is associated with the inhibition of pathogens in the rhizosphere of plants**.** The production of metabolites such as antibiotics and enzymes in *Pseudomonas fluorescens* and *Bacillus* isolates with a wide range of activities may act as antagonistic factors in reducing the growth of *P. pistaciae.* This has already been shown by other researchers (Haas and Défago, 2005; Lugtenberg and Kamilova, 2009; Raaijmakers *et al*., 2002; Raaijmakers and Mazzola, 2012; Shaad *et al*., 2001). Understanding the population dynamics and diversity of bacteria associated with rhizosphere of pistachio may be useful to improve plant growth due to the synthesis of phytohormones, such as IAA, siderophores, ACC deaminase, solubilizing of Phosphate, and improve-

ment of mineral uptake (Lee *et al*., 2015; Compant *et al*., 2010; Kloepper *et al*., 1989; Asghar *et al*., 2004; Glick *et al*., 2007) as well as biotic and abiotic stresses, in particular. Studies have been carried out on the effects of salinity on the growth of different fungal pathogens. For example, Boumaaza *et al.* (2015) showed that the salinity (NaCl and $CaCl_2$ concentrations) stimulated the growth of *Botrytis cinerea* isolates up to 150ppm and higher concentrations reduced the mycelial growth and inhibit spore germination while increasing the formation of sporangia in all concentrations. Benyahyia (1998) demonstrated that increasing the salinity promotes *in vitro* mycelial growth of *P. citrophthora* and *P. parasitica*, the causal agents of root rot of citrus. In view of our findings, no effects was observed at low concentrations on the mycelial growth of *P. pistaciae* (4 and 8 dS/m), while higher salinity (higher than 8 dS/m) reduced the growth to different degrees compared to the control. More research is required to understand the native mechanisms involved in biological control, the effects of specific ions and their concentrations on the growth and sporulation of *Phytophthora* spp. as well as the severity of crown and root rot of pistachio trees under natural conditions in pistachio orchards. Recent observations have shown that the applications of calcium sulfate in pistachio orchards could suppress the incidence and severity of crown and root rot of pistachio trees. Several studies have showed that applications of calcium salts can suppress diseases caused by several pathogens (Biggs *et al*., 1997; Conway *et al*., 1992; Volpin and Elad, 1991; Yamazaki and Hoshina, 1995).

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