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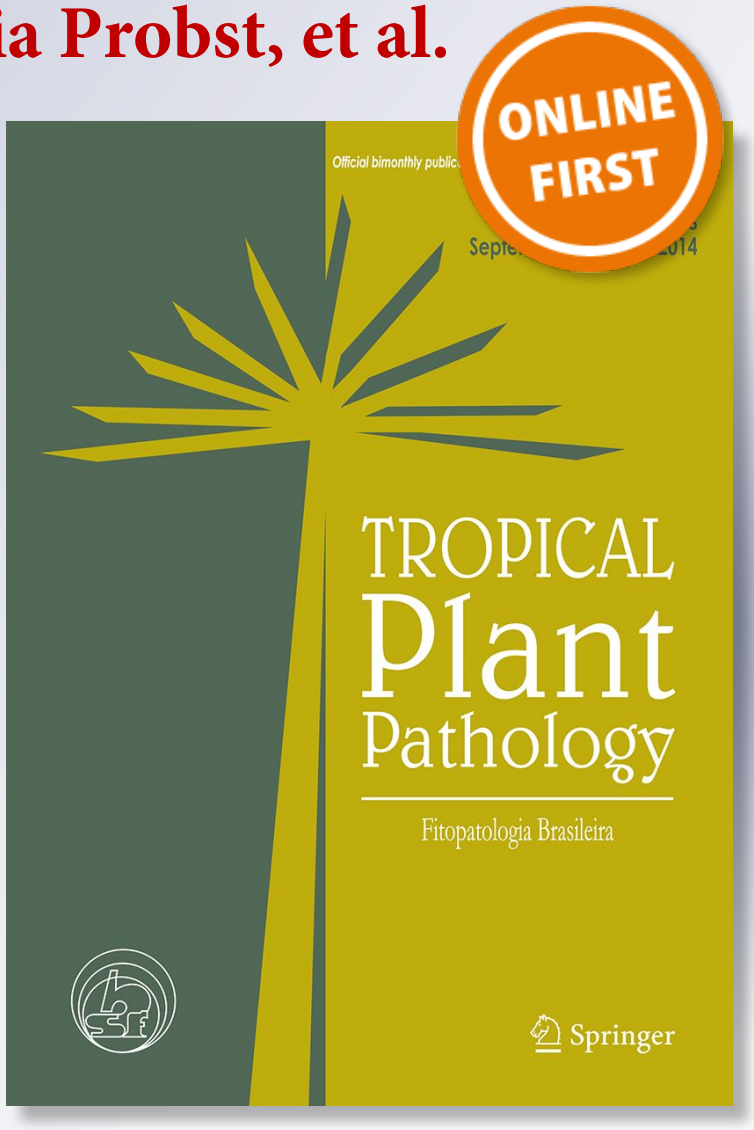


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ORIGINAL ARTICLE

# Efficacy of *Bacillus subtilis* native strains for biocontrol of *Phytophthora* crown and root rot of pistachio in Iran

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## Abstract

In Iran, *Phytophthora* crown and root rot of pistachio trees (also known as gummosis) destroys a significant number of fertile and non-fertile trees each year. To identify potential biocontrol agents effective in controlling pistachio gummosis, 200 soil samples were collected from 19 pistachio growing regions in Kerman province. Out of the 321 strains tested for antagonistic activity against *Phytophthora pistaciae*, 13 were selected as potential inhibitors of the disease. The tested strains were able to inhibit *Phytophthora* growth in dual culture, volatile and non-volatile assays by 14–72%, 12–68% and 27–85%, respectively. The highest inhibition was achieved by three strains identified as *Bacillus subtilis* using phenotypic characteristics, biochemical and physiological tests as well as sequencing the 16S rRNA genomic region for each strain. Co-inoculation experiments of six months old pistachio seedlings with *P. pistaciae* and the three selected *B. subtilis* strains reduced mortality rates by up to 80%. *B. subtilis* strain BSIPR35 was identified as the most promising biocontrol agent in greenhouse experiments. The ability of the selected strains to withstand drought, high temperature and salinity stresses was further tested. The growth of the strains was reduced under abiotic stresses ranging from 22 to 94%. All strains had the same growth under drought stress, while in salinity and under high temperature strain BSIPR35 acted superiorly compared to the other two strains. The bacterial strains may be effective in inhibiting gummosis *in vivo*, which requires further investigations.

**Keywords** *Bacillus subtilis* · *Phytophthora* · Biological control · Gummosis · Pistachio · Root and crown rot

## Introduction

Pistachio (*Pistacia vera*) production in Iran is constantly affected by biotic and abiotic factors. Among them, crown and root rot disease (also known as gummosis), soil salinity, drought and high temperature fluctuations are the most important factors, causing remarkable crop damage each year. Several species within the genus *Phytophthora* have been reported to cause gummosis in Iran (Banihashemi 1994; Mirabolfathy et al. 2001; Fani et al. 2006; Mostowfizadeh-Ghalamfarsa et al. 2008). *Phytophthora pistaciae* and *P. drechsleri* are the species most frequently found in pistachio orchards (Mirabolfathy et al. 2001; Fani et al. 2006). Gummosis can reduce the number of trees by up to 80% over a 5 to 10 year period (Moradi et al. 2017) and, consequently, has an enormous impact on pistachio production. The yearly damage caused by the disease has been estimated to range between 2 and 11% (Moradi et al. 2017). Monoculture cropping, traditional cultural practices in combination with

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abiotic factors such as high soil moisture content and temperature make pistachio cultivars more susceptible to crown and root rot disease and have led to devastating disease epidemics.

Several strategies have been recommended to control disease incidence and severity, such as resistant rootstocks, cultural practices, chemical pesticides and biological control (Erwin and Ribeiro 1996).

Studies have been conducted on screening the antagonistic effects of bacterial strains against *Phytophthora* species. The most important species of bacteria with antagonistic effects to *Phytophthora* are *Streptomyces* and *Bacillus* (Lee et al. 2008; Sadeghy et al. 2014). Among 41 strains of *Bacillus subtilis* obtained from the rhizosphere of a red pepper planting, two strains were able to reduce the disease severity of *Phytophthora capcisi*, the causal agent of pepper blight, by 71 and 86.8%. These strains were also able to promote growth of plant roots and stems (Lee et al. 2008). *In vitro* screenings of 200 *Streptomyces* strains isolated from citrus orchards in Kerman (Iran) showed 20 strains with high antagonistic activity against *P. parasitica* and *P. citrophthora* (Sadeghy et al. 2014). In another study on 130 *Streptomyces* isolates obtained from pistachio orchards in Kerman, 16 isolates inhibited *P. drechsleri* under *in vitro* and *in situ* conditions (Shahidi Bonjar et al. 2006).

Pistachio production is also impacted by abiotic stresses such as drought, high temperature and soil salinity. Therefore, efforts have been applied to minimize these losses. These abiotic stresses adversely affect plant growth and productivity, hence it is important to increase crop tolerance to such abiotic factors (Mahajan and Tuteja 2005). On the other hand, establishment and survival of biocontrol agents have their own challenges in orchards under biotic and abiotic stress conditions. Successful deployment of *Bacillus* strains in stressed ecosystems such as those caused by high temperatures, salt stress, mineral deficiency and heavy metal toxicity depends on their ability to withstand and proliferate under these conditions (Praveen Kumar et al. 2014).

There is not much information on the effects of beneficial *Bacillus* strains native to the soil and rhizosphere of pistachio trees in Iran. Therefore, the present study was conducted to assess the incidence of native *Bacillus* strains in Iranian pistachio production areas and to assess the ability of isolated *Bacillus* strains as potential biological control agents to inhibit *P. pistaciae* in both *in vitro* and *in vivo* conditions. Results of this study can ultimately be used to mitigate pistachio gummosis and consequently prevent yield losses.

## Materials and methods

A virulent *P. pistaciae* strain, originally isolated from infected pistachio trees in Kerman province, was obtained from the Iranian Research Institute of Plant Protection (IRIPP) culture collection. The strain was maintained on either potato dextrose agar (PDA; Merck) or corn meal agar (CMA; Himedia).

### Sampling and bacterial strain isolation

A total of 200 samples were obtained from either soil or the rhizosphere in pistachio orchards located across Kerman province during 2010 to 2012 (Table 1). In each orchard, a diagonal sampling pattern was used. Soil samples ($n = 24$) were collected using a soil auger (60 cm depth). Additionally, the rhizosphere of three randomly selected trees in each orchard was sampled. Samples were stored in labeled propylene bags and were immediately cooled until transferred to the laboratory for analysis. In the laboratory, soil from the rhizosphere was carefully collected from root surfaces using a brush. Soil samples were then thoroughly homogenized and passed through a 2-mm sieve to obtain three 100 g sub-samples. Samples were collected from all sampling areas in both infected and healthy pistachio orchards. Bacteria were isolated from orchards with a soil pH between 6.8 and 8.2, EC = 1.1–12, irrigation periods of 30–60 d, and low organic matter under hot dry conditions. To isolate *Bacillus* strains, the initial soil sample was serially diluted with concentrations ranging from $10^{-1}$ to $10^{-6}$ g of soil. Suspensions of serially diluted samples were heated at 60 °C by placing the flasks in a preheated water bath for 20 min. An 0.1 mL aliquot of each dilution was evenly spread on nutrient agar (NA) medium using a rotating platform (Himedia). Each dilution was spread twice. The plates were incubated at 30 °C for 48 h and examined for bacterial growth. Selected colonies were re-streaked onto fresh NA plates to obtain single, pure colonies.

### Strain identification and PCR conditions

Three selected strains were identified as described in Table 2 based on morphological and biochemical characteristics (Shaad et al. 2001) as well as 16S ribosomal RNA gene sequence analysis (Chen et al. 2010). Total genomic DNA (50 μL final volume) was extracted from each colony using the DNPTM DNA extraction kit (CinnaGen). The 16S rRNA genetic region was amplified using the universal primer set Forward 27 (5′-AGA GTT TGA TCC TGG CTC AG-3′) and Reverse 1492 (5′-GGT ACC TTG TTA CGA CTT-3′). The thermocycler (MJ mini; BioRad) protocol was 94 °C for 4 min, 30 cycles of 94 °C for 60 s, 65 °C for 45 s and 72 °C for 60 s, with a final extension for 10 min at 72 °C. PCR reactions were carried out in a total volume of 25 μL. Each reaction contained 10 μL of 2× master mix (CinnaGen) which includes dNTPs and Taq polymerase, 0.5 μL of each primer, 2 μL of DNA and 12 μL of $dH_2O$. PCR amplification products were prepared for gel electrophoresis (containing DNA safe stain; SinClon BioScience) by mixing 8 μL of the PCR products with the loading buffer.

**Table 1** Characteristics of *Bacillus* strains in Kerman province, Iran

| No. | Sampling location | Number of isolated *Bacillus* | *Bacillus* antagonistic against *Phytophthora pistaciae* | | |
|---|---|---|---|---|---|
| | | | Number | Percentage (%) | Inhibition range* |
| 1 | Tajabad | 10 | 2 | 0.6 | +++ |
| 2 | Kaboutarkhan | 28 | 7 | 2.18 | + |
| 3 | Sharifabad | 20 | 9 | 2.8 | +++ |
| 4 | Esmailabad | 16 | 6 | 1.86 | ++ |
| 5 | Chahjafar | 14 | 4 | 1.24 | ++++ |
| 6 | Khalilabad | 20 | 6 | 1.86 | ++ |
| 7 | Feizabad | 15 | 5 | 1.5 | +++ |
| 8 | Nough | 38 | 13 | 4 | ++++ |
| 9 | Anar(Rezaiyeh) | 19 | 7 | 2.18 | +++ |
| 10 | Kamalabad | 13 | 2 | 0.6 | ++ |
| 11 | Aliabad | 16 | 4 | 1.24 | + |
| 12 | Hamidabad | 17 | 7 | 2.18 | ++++ |
| 13 | Zarand | 33 | 5 | 1.5 | ++ |
| 14 | Hemmatabad | 23 | 8 | 2.5 | ++++ |
| 15 | Naserieyeh | 7 | 1 | 0.3 | +++ |
| 16 | Ferdousiyeh | 6 | 1 | 0.3 | ++ |
| 17 | Lahijan | 26 | 9 | 2.8 | + |
| 18 | Total | 321 | 92 | 28.66 | |

* +, inhibition of mycelium growth by 10–30%; ++, inhibition of mycelium growth by 31–40%; +++, inhibition of mycelium growth by 41–50%; ++++, inhibition of mycelium growth by 60–80%

The mixture was then added to a 1% agarose gel made with 0.5× TAE buffer. Gel electrophoresis was run for 45 min under constant current (45–50 mA) and amplification products were viewed using a UV-transilluminater (Geldocsystem; Gentech). PCR products were sequenced using the original primers of the amplified gene product (Bioneer Sequencing Service, South Korea). Similarity searches of the sequences were carried out using the BlastN option of the NCBI nucleotide database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Table 2** Biochemical and morphological characteristics of *Bacillus subtilis* strains BSIRP7, BSIRP22 and BSIRP35

| Characteristic | Results | Characteristic | Results |
|---|---|---|---|
| Gram staining | + | Methyl red test | + |
| Motility | + | Hydrolysis of tyrosine | – |
| Cell shape | Rod | Formation of $H_2S$ | – |
| Spore formation | + | Litmus milk | + |
| Catalase | + | Hydrolysis of casein | + |
| Growth | | Utilization of propionate | – |
| Anaerobic | – | Utilization of citrate | + |
| Temperature | 45 | Acid production from: | |
| pH | 6.8–7.5 | D-Glucose | + |
| NaCl (7%) | + | Sucrose | + |
| Liquefaction of gelatin | + | Glycerol | + |
| Starch hydrolysis | + | D-xylose | + |
| Nitrate reduction | + | Maltose | + |
| Indole production | – | Lactose | + |
| Urease | + | Fructose | + |

## *In vitro* screening of bacterial antagonistic activity

The ability of the isolated bacteria to reduce mycelial growth of *P. pistaciae* was assessed with the help of dual culture assays. For this, a 5 mm agar plug of a 3-day old *P. pistaciae* isolate was placed in the center of a Petri dish containing CMA. A 5 μL aliquot of an actively growing suspension of each bacterial strain was streaked out on the opposite site of the fungal disc. Petri dishes were incubated at 25 °C in the dark and inhibition efficacy of radial fungal growth noted after 7 d (Leelasuphakul et al. 2008). Bacterial strains showing the greatest inhibitory potential were further selected and analyzed in dual culture, non-volatile, and volatile assays, as described below.

## Effects of non-volatile compounds on mycelial growth of *P. pistaciae*

Twenty-four hour old cultures of bacterial strains were added (200 μL of bacterial suspension at $1 \times 10^7$ CFU/mL) to individual flasks containing 50 mL potato dextrose broth (PDB; Himedia). Flasks were placed on a rotary shaker (150 rpm) at 26 °C for 4 d to promote bacterial growth. Bacterial

suspensions were filtered through Whatman no. 1 filter paper (Whatman; Sigma-Aldrich), and centrifuged at 8000 rpm for 20 min. The autoclaved supernatant was added to sterilized PDA at a ratio of 25:75 (*v*/v) to prepare modified PDA (m-PDA) plates. A 5 mm agar plug of an actively growing *P. pistaciae* isolate was placed in the center of a Petri dish containing the m-PDA medium and incubated at 27 °C in the dark. The mycelial growth was monitored every 48 h for 10 d. The experiment was repeated twice with three replications per treatment. The growth of *P. pistaciae* on regular PDA was used as a control.

## Effects of volatile compounds on mycelial growth of *P. pistaciae*

The assay was conducted according to the method described by Hora and Baker (1972). Briefly, bacterial strains were streaked out on PDA plates and incubated for 3 d at 27 °C. A 5 mm agar plug of an actively growing *P. pistaciae* isolate was placed in the center of the incubated PDA dish, which subsequently was placed upside down on a Petri dish containing a bacterial strain. The construct was sealed with parafilm. As a control, a PDA plate without the pathogen was used as the upper plate. The Petri dishes were stored for 10 d at 27 °C in the dark and mycelial growth was monitored every 48 h. The experiment was repeated twice with three replications per treatment. The ability of bacterial strains to inhibit mycelial growth was calculated.

## Screening of antagonistic effects in greenhouse experiments

The ability of three selected *B. subtilis* strains (BSIPR7, BSIPR22 and BSIPR35) to reduce *Phytophthora* crown and root rot disease severity under greenhouse conditions on six-month old pistachio seedlings was assessed. For this, pistachio seeds (cv. Sarakhs) were planted in 5 L pots (10 seeds per pot) filled with a mixture of sterilized sand:clay (2:1; v/v). After seedling emergence, plants were thinned to four seedlings per pot and grown under greenhouse conditions with a 12 h light photoperiod at 27 ± 2 °C. Inoculum of *P. pistaciae* was prepared as follows. Clean wheat seeds (100 g) were soaked in glass flasks containing tap water for 24 h. After soaking, excess water was removed and seeds were autoclaved in the flasks three times at 1.5 atm pressure and 121 °C for 20 min on three consecutive days. Five agar plugs containing three day old mycelium of *P. pistaciae* were added to each flask and incubated for three weeks at 28 °C in the dark. The flasks were gently shaken every three days.

Bacterial inoculum was prepared using a 24 h old, pure culture of the respective strain. Cultures were added to a flask containing PDB and shaken at 100 rpm for one day. The obtained suspension was centrifuged for 10 min at 3500 rpm. The bacterial concentration was adjusted to $10^9$ (CFU/mL) using sterile distilled water.

The competitive ability of the three selected bacterial strains was assessed in simultaneous inoculation experiments with *P. pistaciae*. The seedlings were inoculated either with bacterial strains and pathogen alone or in two-party mixtures. Ten grams of colonized wheat seeds were placed around the root system of pistachio seedlings and subsequently covered with sterile sand. Thereafter, *Bacillus* strains were poured to the seedling by watering the plants with 200 mL bacterial solution ($2 \times 10^8$ CFU/g) (Lee et al. 1999). Immediately after the inoculation, the pots were flooded for 12 h. After this stage, the excess water was allowed to drain out of the pots as described by Erwin and Ribeiro (1996). The pots were kept under a 12 h photoperiod. Forty-five days after inoculations, seedlings were uprooted and the efficacy of *Bacilli* strains was assessed. The frequency of seedling mortality was evaluated *via* visual assessments and by culturing small pieces of the root and crown on PARP+CMA medium (Masago et al. 1977) with some modifications (pimaricin 10 mg/L, ampicillin 250 mg/L, rifampicin 10 mg/L, terrachlor (PCNB) 75% WP 100 mg/L in 1 L corn meal agar). Plant height, fresh and dry weight of shoots and roots were measured for each seedling and replication. In all inoculations, each treatment consisted of four replicated pots, with four plants in each pot.

## Abiotic stress tolerance

The ability of the three *B. subtilis* strains (BSIPR7, BSIPR22 and BSIPR35) to withstand environmental stresses such as drought (−1.2 Mpa), high temperature (50 °C) and salinity were tested in tryptone soy broth (TSB) by measuring optical densities using a spectrophotometer (T60 U; PG Instruments) at 600 nm. A non-inoculated medium was included in the experiments as a control. Strains with an optical density higher than 0.1 were considered tolerant to abiotic stresses (Praveen Kumar et al. 2014). To assess drought tolerance TSB medium was amended with 32.6% polyethylene glycol-6000 (an osmotic pressure of −1.2 Mpa) by heating and stirring on a hot-plate magnetic stirrer device. For salinity stress, TSB medium was amended with NaCl to create an electrical conductivity ranging from 15 to 20 dS/m, the highest measured ratio in pistachio orchards. To characterize high temperature tolerance, bacterial strains were inoculated in TSB medium and incubated at 50 °C for 24 h. A 30 mL aliquot of amended TSB medium was dispensed into 100 mL flasks and autoclaved. *B. subtillis* strains were grown overnight in TSB medium in a shaker incubator (120 rpm). A 0.1 mL aliquot was used for inoculation and flasks were incubated at 28 °C in a shaker incubator (120 rpm) for 24 h. Bacterial growth was estimated by

recording optical densities at 600 nm using a spectrophotometer. The experiments were carried out in triplicates.

### Statistical analysis

The average values of inhibition of mycelial growth, mortality, plant height, fresh and dry weight of root and shoots were separately determined for each replication. Mean comparisons were made using Duncan's new multiple range test at 0.05 probability. If needed, data were log-transformed prior to analysis.

## Results

### Identification of strains

Bacterial strains were identified at the genus level based on biochemical and morphological features. The three *Bacillus* strains with the highest antagonistic potential were identified as *B. subtilis* (Table 2) by sequencing the 16S rRNA genomic region for each strain. The 16S rRNA gene sequence exhibited a 99% similarity to *Bacillus subtilis* and *Bacillus amyloliquefaciens* (GenBank accession numbers MG203915, MG255306 and MG255307). Furthermore, the BSIPR7, BSIPR22 and BSIPR35 were also identified as *B. subtilis* according to physiological and biochemical tests.

### Antifungal screening of bacterial strains

*Bacillus* isolates were dominant in the orchards of Hamidabad, Lahijan, Sharifabad, Nough and Hemmatabad, while in some locations such as Naserieyeh, Tajabad, Chahjafar, Aliabad, and Feizabad they were found at low frequencies (Table 1). The highest incidences of antagonistic *Bacillus* isolates were found in pistachio orchards of some counties such as Lahijan (80%) and Hemmatabad (82%), while the lowest incidence was found in Ferdousiyeh (12.5%).

Out of 321 *Bacillus* strains, 92 strains (28%) were able to inhibit the growth of *P. pistaciae* in dual culture assays, although to different degrees (Table 1). Among these 92 *Bacillus* strains, 13 strains showed a high ability to inhibit *P. pistaciae* mycelial growth. In-depth evaluations of these 13 *Bacillus* strains revealed different degrees of inhibitory activities against *P. pistaciae* reducing mycelial growth in the ranges 14–72%, 12–68%, and 27–85% in dual culture, volatile and non-volatile assays, respectively (Table 3). Among these 13 strains, isolates BSIRP35, BSIRP22, and BSIRP7 exhibited the greatest inhibitory effect on mycelial growth of *P. pistaciae* compared to other strains, with ratios greater than 64, 62 and 75% for non-volatile, volatile and dual culture assays, respectively, and therefore they were chosen for further studies.

**Table 3** Effects of *Bacillus* spp. strains on the mycelium growth of *Phytophthora pistaciae* in volatile, non-volatile and dual culture assays

| Strain | Sampling Area | Inhibition (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Volatile* | | Non-volatile | | Dual culture | |
| BSIPR79 | Tajabad | 39.3 | c | 36.0 | b | 50.3 | c |
| BSIPR68 | Kaboutarkhan | 14.0 | g | 12.0 | f | 27.0 | h |
| BSIPR22 | Chahjafar | 68.3 | ab | 63.8 | a | 80.0 | ab |
| BSIPR33 | Esmailabad | 29.3 | de | 26.2 | e | 40.0 | de |
| BSIPR62 | Sharifabad | 37.3 | c | 34.0 | bc | 48.3 | c |
| BSIPR44 | Khalilabad | 24.8 | e | 28.3 | de | 39.0 | def |
| BSIPR21 | Feizabad | 41.3 | c | 34.3 | bc | 49.3 | c |
| BSIPR4 | Aliabad | 15.7 | gf | 12.9 | f | 29.5 | gh |
| BSIPR86 | Anar | 35.7 | cd | 33.3 | bcd | 45.2 | cd |
| BSIPR12 | Kamalabad | 22.3 | ef | 27.7 | de | 33.0 | fgh |
| BSIPR7 | Nough | 62.2 | b | 63.8 | a | 75.3 | b |
| BSIPR35 | Hamatabad | 72.3 | a | 68.3 | a | 85.0 | a |
| BSIPR96 | Zarand | 24.3 | e | 29.7 | cde | 35.0 | efg |

*Means followed by the same letter in each column are not significantly different ($p < 0.05$) according to Duncan's new multiple range test

### Competitive ability between *Bacillus subtilis* and *Phytophthora pistaciae* on pistachio seedlings

*Bacillus subtilis* strain BSIPR35 had the greatest potential to reduce gummosis disease in greenhouse experiments (Fig. 1). Control seedlings inoculated with *P. pistaciae* alone had a 100% mortality. *B. subtilis* strains reduced seedling mortality significantly ($p \leq 0.0001$) by 80% (strain BSIPR35), 67% (strain BSIPR22) and 54% (strain BSIPR7). Additionally, *Bacillus* in inoculations alone or in co-inoculation experiments with *P. pistaciae* improved the growth of pistachio seedlings compared to plants inoculated with *P. pistaciae* alone. The effect of *B. subtilis* strain BSIPR35 was most pronounced. The height, dry weight of roots and shoots of plants inoculated with BSIPR35 alone was 1.4 to 1.9 times higher than the one of the control plants (no inoculation). Also, plants inoculated with BSIPR35 had a greater root density (Fig. 2), which may indicate the PGPR effects of this strain. In most cases there were no significant differences between BSIPR22 and BSIPR7 as well as in control plants and *P. pistaciae* alone.

### Abiotic stress tolerance

The growth of the three selected bacterial strains was reduced under abiotic stresses (drought, salinity and temperature), from 22 to 94% (Fig. 3). All strains displayed the same growth rate under drought stress, but under high salinity and under high temperature growth of strain BSIPR35 was superior compared to strains BSIPR22 and BSIPR7. Increasing salinity from 15 to 20 ds/m reduced bacterial growth by 42%, 70 and

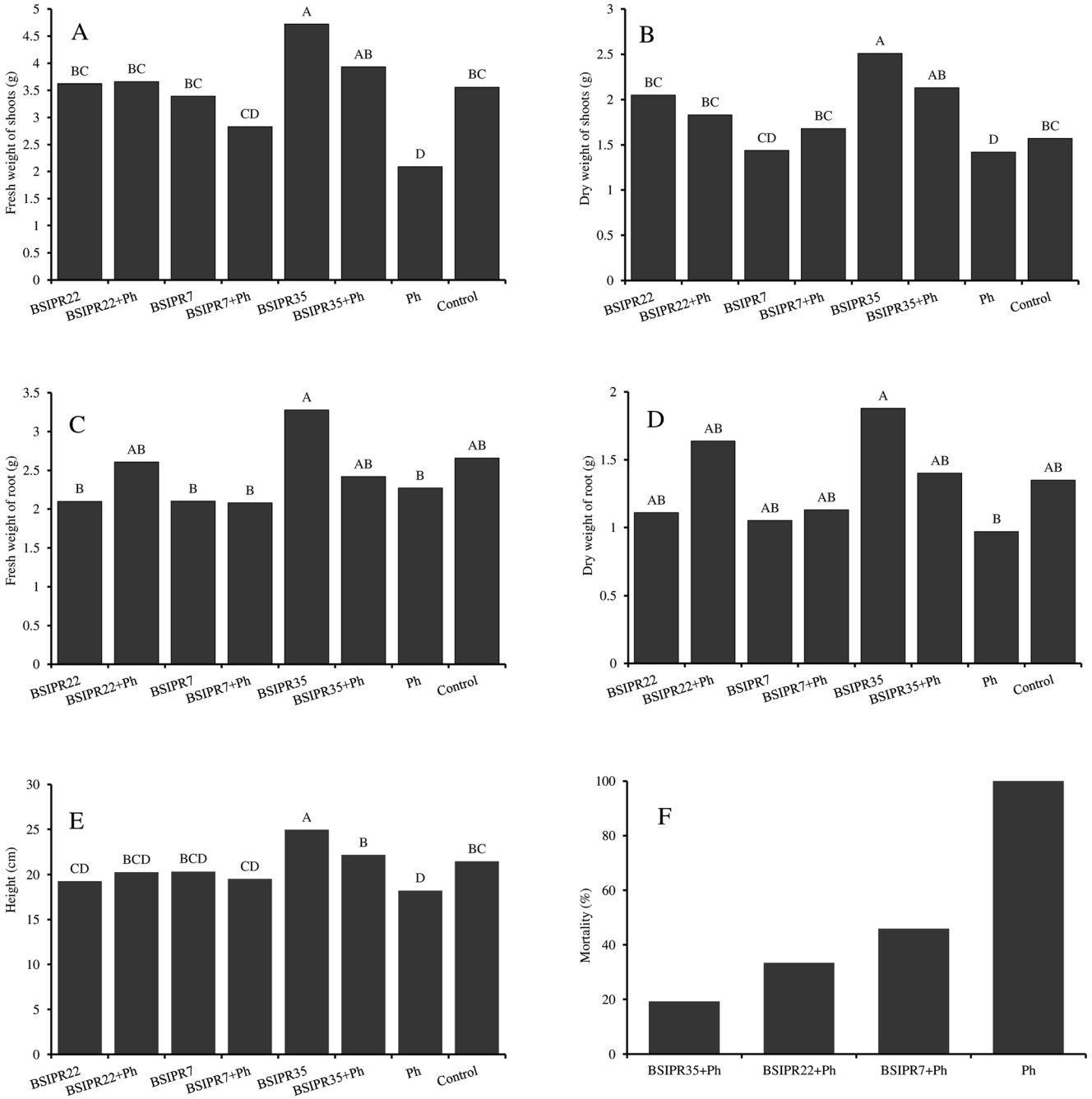


**Fig. 1** Effect of inoculation with *Bacillus subtilis* on mortality and growth characteristics of pistachio seedling cultivar Sarakhs in the presence or absence of the pathogen. **a**, **b**, Fresh and dry weight of shoots. **c**, **d**, Fresh and dry weight of roots. **e**, Height and **f**, Mortality of pistachio seedlings. Bs: *Bacillus subtilis*; Ph: *Phytophthora pistaciae*. Bars with the same letter are not significantly different ($p < 0.05$) according to Duncan's new multiple range test

88% for isolates BSIPR7, BSIPR35 and BSIPR22, respectively. Strain BSIPR 22 was unable to grow at 50 °C.

## Discussion

The use of native bacterial communities is highly preferable when biological control is the desired disease management strategy. These native strains are well adapted to regional environmental and orchard conditions and able to thrive and survive. Screening of pistachio producing areas in the province of Kerman, Iran, indicated the presence of native bacterial strains in soil or in the rhizosphere of pistachio orchards. The potential biocontrol activity of these strains was assessed and confirmed against the causal agent of pistachio gummosis, *P.*


**Fig. 2** Effect of *Bacillus subtilis* strain BSIPR35 on growth and disease progression of pistachio seedlings in contrast to the positive control treatment (infected only with *Phytophthora pistaciae*)

*pistaciae*. Since the isolated *B. subtilis* strains are already well adapted to biotic and abiotic stresses (heat, salinity, and drought) found in pistachio orchards, they may have a higher stability in controlling pistachio gummosis than commercially available biological control products that utilize *Bacillus* strains isolated elsewhere. However, more investigations are required to understand establishment and the dynamics of these populations in soil and/or the plant rhizosphere (Lee et al. 2015).

In general, the incidence of gummosis in pistachio orchards is highest in aging and fruit bearing trees. It has been observed that disease symptoms are most evident at this stage. This could be a result of changing population dynamics and the overall microbial diversity found in the rhizosphere during tree aging. In ginseng, a six year long study has shown that aging reduced the population of *Bacillus* and *Pseudomonas* in the rhizosphere and consequently decreased disease suppression (Li et al. 2014). In cotton, investigations have shown that bacterial diversity

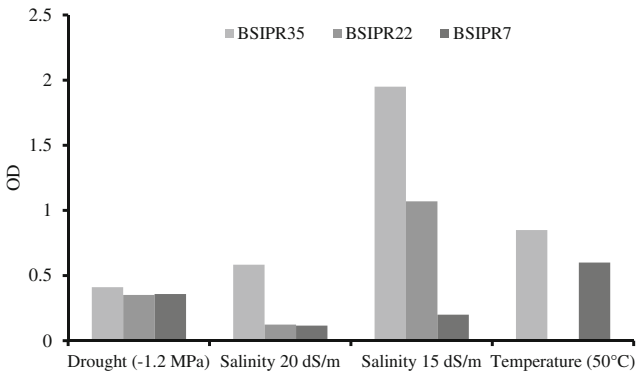


**Fig. 3** Growth patterns of *Bacillus subtilis* under abiotic stresses measured by optical density at 600 nm

increased from budding to flowering stages (Zhang et al. 2011). This may be due to the leakage of greater amounts of organic matter in younger roots, which, in turn, affects the population structure and diversity of microorganisms.

Several studies have shown that the majority of microorganisms found in the rhizosphere are able to improve plant growth, and are referred to as plant growth promoting rhizobacteria (PGPR) (Kloepper et al. 1989; Compant et al. 2005; Lee et al. 2015). Plant responses are due to the synthesis of phytohormones, in particular indole-3-acetic acid (IAA), siderophores, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase by antagonistic bacteria, which stimulate the activities of specific enzymes (Glick et al. 2007). Phosphate-solubilizing bacterial strains increase root ramification, and thus improve mineral uptake. These findings also confirm earlier studies regarding the production of IAA, synthesis of phytohormone, ACC deaminase, and phosphate-solubilizing potential by *Bacillus* and their role in improving plant growth (Glick et al. 2007). Potential of root colonization is a positive aspect in the biological control of soilborne plant pathogens. Cook et al. (1995) reported that the bacteria isolated from the rhizosphere of certain commodities showed better disease control than the microorganisms isolated from other commodities. *Bacillus* species are the most frequently found bacteria in the rhizosphere of many plant species and have been shown to improve plant growth and enhance yield as well as reduce disease severity *via* multiple mechanisms (Compant et al. 2005; Lee et al. 2015).

In our study, *B. subtilis* strain BSIPR35 was the most effective in inhibiting *P. pistaciae* in all *in vitro* assays. This may be due to the production of metabolites such as antibiotics and enzymes with a wide range of activities and overlapping effects that act as antagonistic factors. Similar results have already been reported for different *Bacillus* strains (Shaad et al. 2001; Raaijmakers et al. 2002; Haas and Défago 2005; Lugtenberg and Kamilova 2009; Raaijmakers and Mazzola 2012). Comparisons between the *in vitro* and *in situ* results indicate a higher efficacy of *Bacillus in situ*. It seems that multiple mechanisms are involved in *Phytophthora* inhibition rather than a single factor. These mechanisms may enhance suppression of *Phytophthora* in greenhouse experiments. Similar results have been reported for biocontrol strategies of damping-off in alfalfa by a *B. subtilis* strain. This strain did not have inhibitory effects on *P. megasperma* f. sp. *medicaginis* under *in vitro* conditions, but completely inhibited damping-off under greenhouse conditions (Handelsman et al. 1990). Interaction between *P. pistaciae* and *B. subtilis* strains may explain these contradictory results. Generally, *in vitro* conditions, which frequently only use the mycelium of the pathogen, are used to determine the potential activity of biocontrol agents in different assays. However, under natural conditions zoospores and occasionally the direct germination of sporangia are the

most frequently encountered propagules (Ciancio and Mukerji 2008). Production of volatile and non-volatile compounds are also factors able to inhibit pathogens in the rhizosphere of plants (Raaijmakers et al. 2010). Studies on *Phytophthora* confirmed that zoospores are more sensitive to different compounds compared to oospores, mycelium or cysts (Veena et al. 2010; Hu et al. 2013). Further investigations are required to understand different aspects of biological control such as the method and timing of application, long-term survival, interaction with other naturally found microbes and their adverse effects on fungal inhibition, bacterial responses to predominant, local abiotic stresses in pistachio orchards, and seasonal population dynamics.

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