Biological Control of *Phytophthora Drechsleri* the Causal Agent of Pistachio Gummosis by *Bacillus Subtilis* (VRU1 Strain) in Green House Condition

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Abstract

Introduction: Plant probiotic bacteria are a group of microorganisms that enhance plant growth and protect plants against many diseases by several mechanisms; they can also be used as biofertilizers.

Materials and Methods: In this study, the ability of *Bacillus subtilis* strains (VRU1 and BS-VRU) to produce siderophore, IAA, and some enzymes, such as cellulase, lipase, protease, as well as their potential biocontrol against *Phytophthora drechsler*i, the causal agent of crown and root rot in pistachios, were evaluated in vitro. In addition, the effects of these bacterial strains on growth parameters, such as fresh and dry weights of shoots and roots, plant height, as well as the biocontrol of *P. drechsleri* were investigated under greenhouse conditions.

Results: According to the results of this study, two strains were able to produce siderophore, IAA, lipase, cellulas, and protease. Greenhouse results showed that both strains of *B. subtilis* increased growth parameters significantly in inoculated plants compared to the control. In addition, the results obtained showed that these bacterial strains decreased the mortality rate of pistachio seedlings, with the highest and lowest reductions having been 75% and 50% in VRU1 and BS-VRU, respectively.

Conclusion: According to the results, pistachio gummosis could be controlled by *Bacillus subtillis* bacteria. In addition, *B. subtilis* VRU1 and BS-VRU showed high efficacy in the suppression of diseases through several biocontrol mechanisms.

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1. Introduction

Pistachios are among the most important horticultural crops cultivated in Iran. Iran has been the major producer and exporter of pistachios in the world with the total production of 575,000 tons in 2017 [1]. Soil-borne diseases are among the most prevalent diseases of pistachios in the world. Gummosis is caused by several species of *phytophthora* and is of economic importance in Iran as well as some parts of the world [2]. Many crop plant diseases are caused by plant pathogens, which result in economic losses. The use of bacterial agents is a great choice for fighting against plant pathogens and an excellent alternative for the use of chemicals. *Pseudomonas* and *Bacillus* genera are the two most common biocontrol agents with significant benefits, such as PGP[1] properties [3]. In recent decades, the use of chemical compounds in controlling the pests and diseases of plants has been declining in advanced countries, due to the awareness of their effects, so non-chemical methods, such as biological control have been more prevalent. The use of plant growth-promoting rhizobacteria is of particular importance because it does not harm the environment and helps improve the quality of crops, thereby maintaining consumer health [4]. These bacteria increase plant growth by various direct and indirect mechanisms [5]. PGPR[2] are associated with plant roots and can enhance the plant yield by mechanisms that help improve inorganic nutrient uptake, phytohormone production, and disease suppression [6]. Among these bacteria, *Bacillus* and *Pseudomonas* have the highest microbial population around the root zone [7]. Plant growth promotion can be defined as the synthesis of antibiotics or other compounds, in the form of Fe reduction in rhizosphere or access to nutrients through P solubilisation, which exerts inhibitory effects on pathogenic organisms in rhizosphere [8, 9]. According to Kilin *et al* (2000), *Bacillus subtilis* increases plant growth through multiple mechanisms, such as competition, root colonization, antibiosis, induction of host plant resistance, as well as production of some compounds, such as auxin, protease, lipase, and cellulose, thereby increasing water absorption and stress tolerance. Iron is an element required for essential cellular activities in almost all microorganisms [10]. Since the bio-access of iron is limited in natural regions, a large number of bacteria produce ferric iron-chelating compounds known as siderophores to increase access to different iron sources [11]. Bacteria belonging to genera *Pseudomonas*, *Rhizobium*, *Xanthomonas*, *Enterobacter*, *Bradyrhizobium*, and *Azetobacter* produce auxin which stimulates plant growth [12]. In addition, the production of cellulases, lipases, and other lytic enzymes by *Bacillus subtilis* can affect plant growth [13]. Hence, this research aims to recognize if *B. subtilis* VRU1[3] and BS-VRU[4] provide an impressive biological control against gummosis caused by *P. drechsleri* in pistachio seedlings.

[1] Plant Growth-Promoting (PGP)
[2] Plant Growth-Promoting Rhizobacteria (PGPR)
[3] Bacillus Subtilis VRU1 (VRU1)
[4] Bacillus Subtilis BS-VRU (BS-VRU)

2. Materials and Methods

2. 1. Preparation of *Microbial Strains*

B. subtilis strains (VRU1 and BS-VRU) were selected from the biological control collections of Vali-e-Asr University of Rafsanjan, and an isolate of *Phytophthora drechsleri* was retrieved from the PRC[5] of Rafsanjan.

2. 2. Siderophore Production

Two strains were screened for siderophore production according to the method of Alexander and Zuberer [14]. Siderophores produced by bacterial strains were detected using the CAS[6] agar medium. Orange halos around the bacterial colonies showed signs of siderophore production.

2. 3. Protease Production

Protease production was measured according to the procedure introduced by Marhofer *et al* [15]. The diameters of colorless halos around the colonies in the SMA[7] medium indicated the protease production ability.

2. 4. Lipase Production

Lipase production by strains VRU1 and BS-VRU was measured through the method described by Omidvari [16], using 0.1 g $CaCl_2$, 5 g NaCl, 10 g peptone, 15 g Agar, 1 liter distilled water, and 10 ml Tween 20 (sterile). Two strains were streaked on this medium and incubated at 28˚C for 72 h. Depositions around the strain colonies indicated the activity of lipase.

2. 5. Cellulase Production

Two strains were screened for cellulase production by adopting the method of Kasana *et al* [17]. Bacterial strains were streaked on CMC agar (0.1% K_2HPO_4, 0.05% KCl, 0.05% $MgSO_4$, 0.2% $NaNO_3$, 0.02% peptone, 0.2% carboxy methyl cellulose, and 1.7% agar) and were incubated at 27˚C for 72 h, with the plates flooded with 0.1% congo red for 15 min and then with 1 M NaCl for 15 min. After 72 h, clear halo zones around the colonies showed the cellulose-producing bacterial ability.

2. 6. Auxin production

Auxin production by bacterial strains was measured according to the method introduced by Patten and Glick [12].

2. 7. Antifungal activity

A mycelial disk (5 mm) of *P. drechsleri* was placed at the center of a petri dish containing a lima bean agar medium, and the bacterial antagonist was streaked at a 0.5-cm distance from the margin of the plates. Next, the plates were incubated for 5 days at 28°C. After the incubation period, the inhibition zone was measured [18].

2. 8. Preparation of a *Phytophthora Drechsleri* Inoculum

To prepare an inoculum, 25 g of rice grains was added to 18 ml of water in a 250 ml Erlenmeyer flask, and then it was autoclaved for 30 min at 121°C on two consecutive days. The flask was inoculated with a six mycelial plug from a three-day-old culture of *P. drechsleri*, incubated three weeks at 25°C in the dark [19].

[5] Pistachio Research Center (PRC)
[6] Chrome Azurol S (CAS)
[7] Skim Milk Agar (SMA)

2. 9. Greenhouse Experiments

The pistachio cultivar of Sarakhs was used in this study. To establish the cultivar in soil, the seeds were kept in sterile distilled water for 24 h, before they were soaked in the mixture of sand and coco peat (1-1, V/V) for germination at 28°C for 7 days [20]. Five germinated seeds were placed in 4-kg pots containing sterile soil, pH 7.6, and EC 3dSm-1. Five g of *P. drechsleri* of colonized rice was added around the roots of two-month-old pistachio seedlings from Sarakhs cultivar. To control the plants, 5g of sterile rice was used. The inoculum of the two strains (VRU1 and BS-VRU) was prepared on the nutrient agar at 28°C for 24 h. The concentration was equal at 4×10^{10} CFU mL-1. The suspension of bacterial strains was prepared in sterile distilled water, with the 40-ml suspension of each bacterial strain added to each pot containing 4 kg of soil. After 45 days, the percentages of disease reduction and plant growth factors (stem length, as well as fresh and dry weights of stems and roots) were examined. The mortality rate was computed as follows [21]:

X= 100– (100× A)/B

Where A is the number of infected plants in each treatment, B represents the number of infected plants in the control group, and X is the mortality rate.

2. 10. Statistical Analysis

The data were analyzed by a one-way ANOVA. In addition, SAS 9.1 (SAS Institute, Inc., Cary, NC, USA) was used to analyze the data and compare the mains.

3. Results

3. 1. Siderophore Production by Bacterial strains

The results of the siderophore production test determined the diameters of orange halos around the bacterial colonies on the CAS agar medium, which were measured after 72 h. The highest siderophore production was recorded for strain BS-VRU at 2.1 cm, and strain VRU1 showed the minimum siderophore production at 1.8 cm.

3. 2. Cellulase Production by Bacterial Strains

The results of the cellulase test showed that VRU1 and BS-VRU were able to produce cellulase. The largest halo around the colonies was formed by BS-VRU. Halo diameters around the colonies of VRU1 and BS-VRU strains were 0.9 mm and 0.7 mm, respectively.

3. 3. Auxin Production by Bacterial Strains

Auxin (IAA)[8] production was observed in the two strains of VRU1 and BS-VRU, which produced 19.3 µg/ml and 11.14 µg/ml of auxin, respectively.

3. 4. Protease and Lipase Production by Bacterial Strains

The results of the lipase test showed that two bacterial strains had a high ability to produce lipase. In addition, VRU1 and BS-VRU were able to produce protease in the SMA medium. The diameters of the halo zone around the bacterial colonies on the SMA medium were measured after 24 h. The maximum protease production by BS-VRU was 2.2 cm. In contrast, VRU1 showed the minimum protease production at 1.9 cm.

[8] Indole Acetic Acid (IAA)

3. 5. Antifungal Activity

The results of the inhibitory zone demonstrated that two bacterial strains inhibited the growth of *P. drechsleri*. The highest and lowest values for the inhibitory zone were obtained by strains VRU1 and BS-VRU, respectively.

3. 6. Greenhouse Experiments

3. 6. 1. Mortality Rate

Seedling mortality rates decreased significantly (P< 0.05) by 75% and 50% in seedlings treated with VRU1 and BS-VRU1, respectively. All pistachio seedlings inoculated only with *P. drechsleri* died after 21 days from inoculation (Fig. 1)

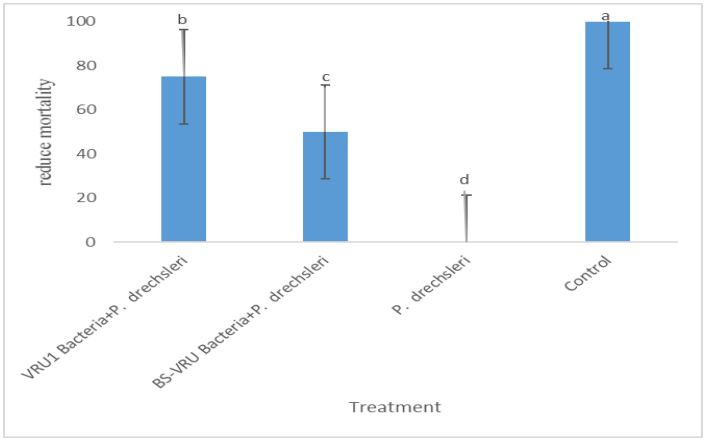

Fig. 1: Effects of bacterial strains on the rate of mortality in pistachio cultivar Sarakhs inoculated with Phytophthora drechsleri in greenhouse experiment

3. 6. 2. Seedling development

According to the results, shoot height was significantly affected by bacterial strains. Higher values were recorded in seedlings inoculated with VRU1 than in seedlings inoculated with BS-VRU. The treatment inoculated with strain VRU1 showed the highest value (P< 0.05) among all other measured parameters. Strains VRU1 and BS-VRU resulted in an improvement in the aforementioned growth factors in seedlings not inoculated and inoculated with *P. drechsleri*, compared to the plants not inoculated with bacteria. In terms of shoot fresh weight, inoculation with VRU1 and BS-VRU alone resulted in 1.33 cm and 0.79 cm, respectively; in addition, when mixed with *P. drechsleri*, it resulted in 1.11 cm and 0.71 cm in strain VRU1 and strain BS-VRU, respectively. According to the results, in the treatment inoculated with strain VRU1, the fresh weight of the root was more than that of the other treatment. Similar results were observed in the dry weight of the roots and shoots. In addition, bacterial strains resulted in more powerful growth parameters than the control (Table 1). The seedlings inoculated with strain VRU1 were more significantly effective than strain BS-VRU.

Table 1. Efficacy of *Bacillus subtilis* (VRU1 and BS-VRU strains) on growth parameters in pistachio seedlings either in inoculation with *P. drechsleri* or alone

Root dry weight (g)	Shoot dry weight (g)	Root fresh weight (g)	Shoot fresh weight (g)	Shoot Length (cm)	Treatment
VRU1 Bacteria	0.99^{a}	0.71^{a}	1.91^{a}	1.33^{a}	13.66^{a}
BS-VRU Bacteria	0.58^{c}	0.61^{b}	0.99^{c}	0.79^{c}	10.66^{c}
VRU1 Bacteria + *P.drechsleri*	0.77^{b}	0.38^{c}	1.43^{b}	1.11^{b}	12.33^{b}
BS-VRU Bacteria + *P.drechsleri*	0.46^{d}	0.34^{cd}	0.92^{c}	0.71^{cd}	8.66^{d}
P.drechsleri	0.09^{f}	0.15^{e}	0.2^{e}	0.31^{e}	6.66^{e}
Control	0.37^{e}	0.3^{d}	0.77^{d}	0.63^{d}	8.66^{d}

Notes: Notes: Means followed by different letters show significant differences at $p<0.05$ (LSD)

4. Discussion

Among diseases of pistachio trees in Iran, gummosis caused by various species of *Phytophthora* is of particular importance, and in the fields with suitable conditions for the pathogen, the crop yield loses its economic value. *Phytophthora* causing crown and root rot is extremely dangerous and leads to the death of trees. Today, due to the environmental pollution crisis caused by the excessive use of pesticides and chemical compounds in controlling plant diseases and pests, human health has been endangered in different societies, so many attempts have been made to find suitable solutions for replacing these compounds and removing pollutants. One of the management strategies for plant diseases is the use of plant growth-promoting rhizobacteria. Plant growth-promoting rhizobacteria exert positive effects on host plants. Various mechanisms are involved in the biocontrol of antagonists, such as enzymes (lipase, cellulose, and the like), siderophore, hydrogen cyanide, as well as in phosphate solubilization. The use of PGPR bacteria has been reported to lead to an improvement in various growth indicators, such as root growth, germination rate, root and shoot weights, as well as the biocontrol of pathogens [22]. The production of phytohormones by PGPR can affect plant development [23, 24]. *Bacillus* genus produces a diversity of hormones, such as auxin that plays an important role in plant development [3]. Idris et al [25] detected high levels of auxin in *B. subtilis* (FZB37) and *Bacillus amyloliquefaciens* (FZB24, FZB42, and FZB45). Asghar *et al* (2002) reported that PGPR strains produced 24.6 mg/ml of auxin, while in the present study, two strains were able to produce auxin, with its maximum quantity having been 19.3 µg ml-1. The effects of *B. subtilis* on auxin production and shoot length indicated a positive correlation between shoot length and the content of auxin

production. The seedlings inoculated with strain VRU1 with the high ability of auxin production resulted in the highest shoot length. Lipase and protease can contribute to the potency of bacteria in inhibiting fungal diseases [26]. A limited reserve of iron and some metals produced by siderophore through chelation could act as biological control for some plant diseases [27]. In general, the production of these components by two strains showed the potential of PGPR for acting as biological control. The cell walls of oomycetes are composed of cellulose. Cellulolytic enzymes are probably useful for the biological control of oomycete pathogens, such as *Phytophthora* [28]. The results of greenhouse experiments showed that strains with the highest ability to produce protease, IAA, lipase, and siderophore could improve plant growth. Strain VRU1 showed less inhibitory effects in the plate assay, but it showed the best control of pathogens in greenhouse experiments. In contrast, strain BS-VRU1 showed the highest inhibitory effect in the plate assay; however, it showed less effects than VRU1 on the control of pathogens in greenhouse experiments (Fig. 2).

Fig. 2: the effect of bacterial strains on growth parameters in pistachio. (A: Bs-VRU bacteria, B: Bs-VRU bacteria + *P. drechsleri*, C: Control, D*: P. drechsleri*, E: VRU1 bacteria, F: VRU1 bacteria + *P. drechsleri)*

5. Conclusion

The research results indicated that pistachio gummosis disease can be controlled with Bacillus subtilis bacteria .B.subtilis strains (VRU1, BS_VRU) showed high efficacy in the metabolite produced and inhibited growth of the pistachio gummosis in greenhouse conditions. However, more research is needed to find the effective formulation for PGPR bacteria to inhibit Phytophthora drechsleri in garden conditions.

Conflict of interest

The authors declare no conflict of interest.

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