An Evaluation of Four Plant Growth-Promoting Rhizobacteria (PGPR) and Their Effects on Controlling Crown and Root Rot of Pistachios Caused by *Phytophthora Parsiana*

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Abstract

Introduction: The use of plant growth-promoting rhizobacteria (PGPR) is a promising method in sustainable agriculture and can be an efficient alternative to chemical pesticides.

Materials and Methods: In this study, antagonistic features of four bacterial strains, including *Pseudomonas fluorescens* VUPF5, *P. fluorescens* T-17, *Bacillus subtilis* V1, and *B. subtilis* B96 were investigated. In addition, their effects on crown and root rot of pistachios (gummosis), caused by *Phytophthora parsiana*, were assessed. Besides, different populations (10^4, 10^6, 10^8 CFU/ml) of bacterial strains on the fresh and dry shoot and root weight of pistachios in the Sarakhs cultivar were evaluated.

Results: The results showed that all bacterial strains had remarkable antagonistic effects and were capable of controlling pistachio gummosis under greenhouse conditions. *P. fluorescens* VUPF5 had the strongest effect among the selected strains under laboratory and greenhouse conditions, with a 70% reduction in the mortality rate. Upon a reduction in the initial inoculated population of the bacteria, their growth-promoting effects on growth factors in pistachio seedlings decreased.

Conclusion: Research should be carried out on the efficiency of the selected rhizobacteria strains by utilizing them under field conditions to evaluate their effectiveness in interaction with other microorganisms.

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1. Introduction

Plant disease control is primarily done based on chemical methods because finding an effective alternative is often difficult. However, resistance to fungicides and public concerns about agricultural chemicals have created the demand for efficient and safe products of low toxicity to humans, wildlife, and the environment with low residues in foods [1]. Microorganisms are promising alternatives able to control phytopathogens and their resulting disease [2]. Among these microorganisms, bacteria living in the rhizosphere can be valuable candidates for biological control of plant diseases.

PGPRs (plant growth-promoting rhizobacteria) are a group of bacteria able to colonize the rhizosphere aggressively and improve plant growth and yield. It is documented that only 1-2% of bacteria in the rhizosphere can promote plant growth [3]. PGPRs exert their beneficial effects directly or indirectly. In the direct manner, the bacterium synthesizes a compound, such as phytohormones, which improves plant health and growth. Besides, such a compound may increase the uptake of some nutrients from the environment. In the indirect manner, PGPRs decrease or prevent destructive effects of phytopathogens by producing substances with antagonistic effects or by inducing resistance to pathogens [4]. Pathogen antagonism and production of hydrogen cyanide are among indirect effects of PGPRs. After applying PGPRs, they colonize the surface and inside of roots, thereby limiting expansion of pathogens on the roots. However, in many PGPRs, nutrient competition, niche exclusion, production of antibiotics, siderophores, and hydrogen cyanide, as well as induction of resistance are the main biocontrol mechanisms [5-8]. It is worth noting that rapid and effective root colonization is the first step in biological control. This is because PGPRs must be able to compete with indigenous microflora, including pathogens [2]. This could be the reason why some PGPR strains, despite yielding satisfactory results *in vitro*, show varied biocontrol efficacies under greenhouse or field conditions.

Several *Phytophthora* species cause crown and root rot (gummosis) in pistachio trees. Gummosis has a damaging impact on pistachio production and can eradicate 80% of pistachio trees in five to 10 years [9]. Given negative ecological effects of chemical methods, the use of biological agents can be a good alternative for controlling gummosis. Among different genera identified as PGPRs, *Bacillus* spp. and *Pseudomonas* spp. are the most studied and well-known groups of rhizobacteria with effects against soil-born fungal pathogens [10]. Many PGPRs are able to reduce *Phytophthora* root and

crown rot in various plants [10- 13]. In addition to pathogens, abiotic stresses, such as soil salinity, high temperature, and drought affect pistachio production adversely [9]. In addition, bacterial strains can be effective in reducing abiotic stresses.

Biological control is highly effective in protecting plants against unfavorable conditions. Accordingly, we screened four rhizobacteria strains *in vitro* and under greenhouse conditions in the Sarakhs cultivar of pistachios. The results of this paper can be used to treat pistachio gummosis and help get aligned with sustainable agriculture.

2. Materials and Methods

2.1. Plant growth-promoting assays

2.1.1. Bacterial strains and the gummosis agent

Four rhizobacteria strains, including *Pseudomonas fluorescens* VUPF5, *P. fluorescens* T-17, *Bacillus subtilis* V1, and *B. subtilis* B96 were retrieved from the culture collection of Vali-e-Asr University of Rafsanjan, Iran. Additionally, *Phytophthora parsiana* was provided from the pistachio research institute of Iran, Kerman, Rafsanjan.

2.1.2. Dual culture assay

Bacterial strains were spotted on the PDA+King's B medium, 1cm from the plate edge. Simultaneously, a 0.5-cm plug from the five-day *Phytophthora parsiana* fungal culture was incubated at the opposite edge of the plates. The plates were incubated at 28^{o}C for 7 days. The inhibition zone was scored by measuring the distance between the edge of the fungal mycelium and the bacterial colony. For the purpose of the control treatment, a plate inoculated only with *P. parsiana* was utilized. This experiment was carried out in three replicates per treatment. The data were analyzed using SAS 9.1. Besides, the mean comparisons were made using the Duncan's new multiple range test at a 5% probability level.

2.1.3. Antifungal volatile compounds

A dual-plate test was performed to estimate antifungal volatile compounds. Next, a 5mm plug of the fungi was placed at the center of a PDA plate. The bacterial strains were streaked into an NA plate. The fungal and bacterial plates were placed against each other and were connected with a tape.

On the dual plates, the fungal culture side was placed at the bottom to prevent contamination of bacterial plates by propagation of fungal growth. In the controls, the media without bacterial strains were used. After incubation at 25^{o}C for 72h, fungal colony diameters (mm) were measured, and the mycelial growth inhibition rate (%) was calculated according to the following formula:

$\frac{A-B}{A} \times 100$ = Mycelial growth inhibition rate (%)

A: Fungal growth diameter (mm) in controls

B: Fungal growth diameter (mm) on treated plates

2.1.4. Protease production

Protease activity was measured by casein degradation in skim-milk agar, in which protease production created a clear zone in the medium [14]. Besides, bacterial strains were cultured on agar plates on a point basis. The formation of a halo zone around the bacterial colony accounted for the positive protease production.

2.1.5. Hydrogen cyanide production

The ability of the selected rhizobacterial strains to produce HCN was assessed according to a method described by Lork [15]. Accordingly, each strain was streaked in a King's B agar medium. Next, Whatman® filter papers that were soaked in a solution containing 0.5% picric acid and 2% sodium carbonate were fixed to the lids of Petri dishes. The plates were enclosed tightly in parafilm and incubated at 27ºC for 7 days. Changes in the color of the filter papers from yellow to reddish-brown were considered as different levels of cyanide production. Besides, a King's B agar medium, without bacterial inoculation, was used as the negative control.

2.2. Determination of fresh and dry shoot and root weight

To plant pistachio seeds, five germinated seeds were sown in 4kg plastic pots filled with sterilized sandy soil [16]. After one month, seedlings were inoculated with four bacterial strains. Bacterial strains, including VUPF5, T-17, V1, and 96 were obtained from the PGPR collection of Vali-e-Asr University of Rafsanjan, Iran. Next, 40ml of 10^4, 10^6, and 10^8 CFU/ml of the bacterial suspension was added to each pot. Regarding the control plants, distilled water was added to the pots. This experiment was performed in the randomized complete block design for 13 treatments and in three replicates per each treatment. After 45 days, the growth factors were measured. Next, the shoots and roots were dried in an oven at 60ºC for 48h to measure dry shoot and root weight. Accordingly, the data were analyzed using SAS 9.1, and the mean comparisons were made using the Duncan's new multiple range test at a 1% probability level.

2.3. Evaluation of gummosis control by bacterial strains

The reduction in the mortality rate caused by bacterial strains was measured in 1-month-old seedlings of the Sarakhs cultivar of pistachios. The seeds were sown in 4kg pots. Next, the pistachio seedlings' roots were inoculated with rice seeds colonized by *Phytophthora parsiana* (5gr of colonized rice per 1kg of pot soil) [16]. At the same time, the bacterial strains were inoculated. In the meantime, the control treatment was inoculated with sterile distilled water and sterilized rice in a pot. The symptoms were recorded and analyzed after three weeks.

3. Results

3.1. Screening antifungal effects of bacterial strains

The dual culture assay showed that all bacterial strains were capable of controlling mycelial growth of *P. parsiana*. Besides, VUPF5 showed a high ability to inhibit *P. parsiana* mycelial growth, and V1 had the lowest ability to do so (Table 1).

Table 1- Average diameter of the inhibitory zone (mm)

Bacterial strain	Average diameter of the inhibitory zone (mm)	Grouping at a 5% probability level
VUPF5	17.6	A
B96	14.4	AB
T-17	14.0	AB
V1	12.6	B

Dissimilar letters show significant differences in the means at 5%.

3.2. Antifungal volatile compound assay

In the antifungal volatile compound assay, VUPF5 showed the highest inhibitory effect (81.7%) against *P. parsiana*. After VUPF5 Strain T-17 with 69.9%, B96 with 53.8%, and V1 with 31.2% showed the highest inhibitory effects.

3.3. Ability to produce protease

VUPF5, T-17, and V1 were capable of producing protease with the average diameter of 4.5mm in the skim milk agar plates, but protease production was not observed in strain B96.

3.4. Production of HCN

According to the results, the strains produced different levels of HCN. Accordingly, VUPF5 and B96 had the maximum and minimum ability to produce HCN, respectively. Besides, the HCN-production ability of *P. fluorescens* T-17 was relatively high, yet that of *B. subtilis* V1 was relatively low.

3.5. Fresh and dry shoot weight

In contrast to the control, all bacterial strains increased fresh and dry shoot weight even in low populations (10^4 CFU/ml). The population of 10^8 in T-17 and VUPF5 strains acted more efficiently in increasing fresh root weight. However, the population of 10^4 in the V1 strain acted less efficiently in doing so. Besides, the highest mean of dry shoot weight belonged to the population of 10^8 in the T-17 strain (Fig. 1 and Table 2).

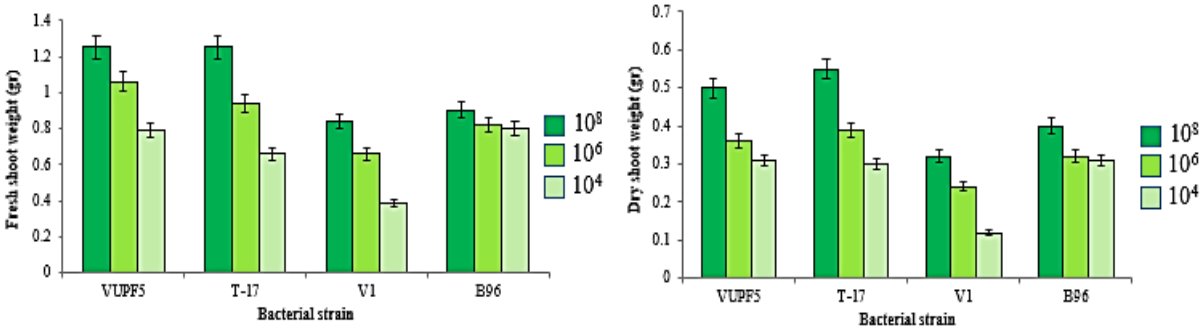

Fig. 1- Fresh and dry shoot weight in different populations of bacterial strains

Table 2-1- Grouping of the data obtained from measuring fresh shoot weight in different populations of bacterial strains

Bacterial Strain	Population	Mean of fresh shoot weight	Grouping at a 1% probability level
T-17	10^8	1/25	A
V5	10^8	1/25	A
V5	10^6	1/06	AB
T-17	10^6	0/94	ABC
96	10^8	0/90	ABC
V1	10^8	0/84	BC
96	10^6	0/82	BC
96	10^4	0/80	BC
V5	10^4	0/79	BC
V1	10^6	0/66	CD
T-17	10^4	0/66	CD
V1	10^4	0/39	DE
Control	_	0/27	E

Table 2-2- Grouping of the data obtained from measuring dry shoot weight in different populations of bacterial strains

Bacterial Strain	Population	Mean of dry shoot weight	Grouping at a 1% probability level
T-17	10^8	0/55	A
V5	10^8	0/50	AB
96	10^8	0/40	BC
T-17	10^6	0/39	BC
V5	10^6	0/36	CD
V1	10^8	0/32	CD
96	10^6	0/32	CD
96	10^4	0/31	CD
V5	10^4	0/31	CD
T-17	10^4	0/30	CD
V1	10^6	0/24	DE
V1	10^4	0/12	EF
Control	_	0/02	F

Dissimilar letters show significant differences in the means at 1%

3.6. Fresh and dry root weight

Strains T-17 and VUPF5 in the population of 10^8 acted more efficiently in increasing fresh root weight. Besides, there were no significant differences between the population of 10^4 from VUPF5, T-17 and B96 strains and between the population of 10^4 and 10^6 from the V1 strain with the control. The measurement of dry root weight showed that the highest mean belonged to the population of 10^8 from B96 and T-17 strains. Besides, there was no significant differences between the population of 10^4 from the V1 strain and the control. In addition, the totally fresh and dry root weight increased upon an increase in the population of bacterial strains (Fig. 2 and Table 3).

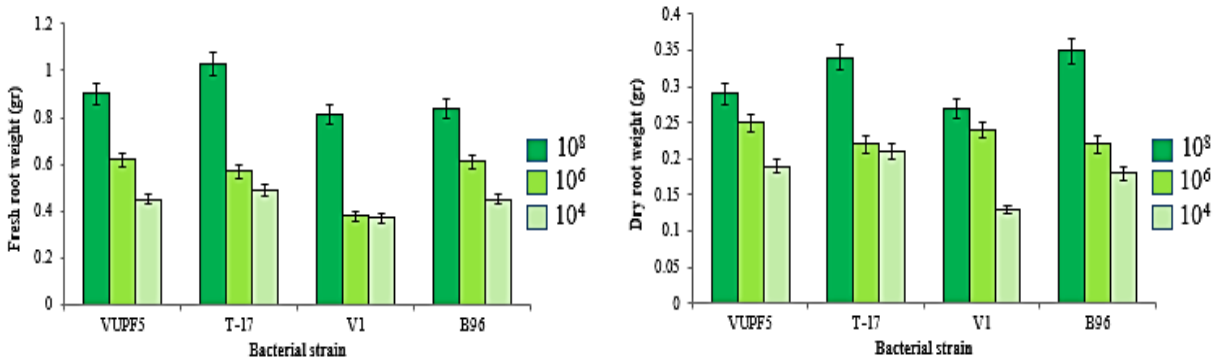

Fig. 2- Fresh and dry root weight in different populations of bacterial strains

Table 3-1- Grouping of data obtained from fresh root weight in different populations of bacterial strains

Bacterial Strain	Population	Mean of fresh root weight	Grouping at a 1% probability level
T-17	10^8	1/03	A
V5	10^8	0/90	AB
96	10^8	0/84	ABC
V1	10^8	0/81	ABC
V5	10^6	0/62	BCD
96	10^6	0/61	BCD
T-17	10^6	0/57	CD
T-17	10^4	0/49	D
V5	10^4	0/45	D
96	10^4	0/45	D
V1	10^6	0/38	D
V1	10^4	0/37	D
Control	_	0/31	D

Table 3-2- Grouping of data obtained from dry root weight in different populations of bacterial strains

Bacterial Strain	Population	Mean of dry root weight	Grouping at a 1% probability level
96	10^8	0/35	A
T-17	10^8	0/34	A
V5	10^8	0/29	AB
V1	10^8	0/27	AB
V5	10^6	0/25	AB
V1	10^6	0/24	ABC
96	10^6	0/22	BC
T-17	10^6	0/22	BC
T-17	10^4	0/21	BC
V5	10^4	0/19	BC
96	10^4	0/18	BC
V1	10^4	0/13	C
Control	_	0/13	C

Dissimilar letters show significant differences in the means at 1%

3.7. Disease control rate

PGPR strains suppressed the growth of the fungus under greenhouse conditions. Among the bacterial strains, VUPF5 reduced the mortality rate by 70%. Besides, T-17 and V1 were at the second place by a 65% reduction, and B96 was at the third place by a 60% reduction in the mortality rate (Fig. 3).

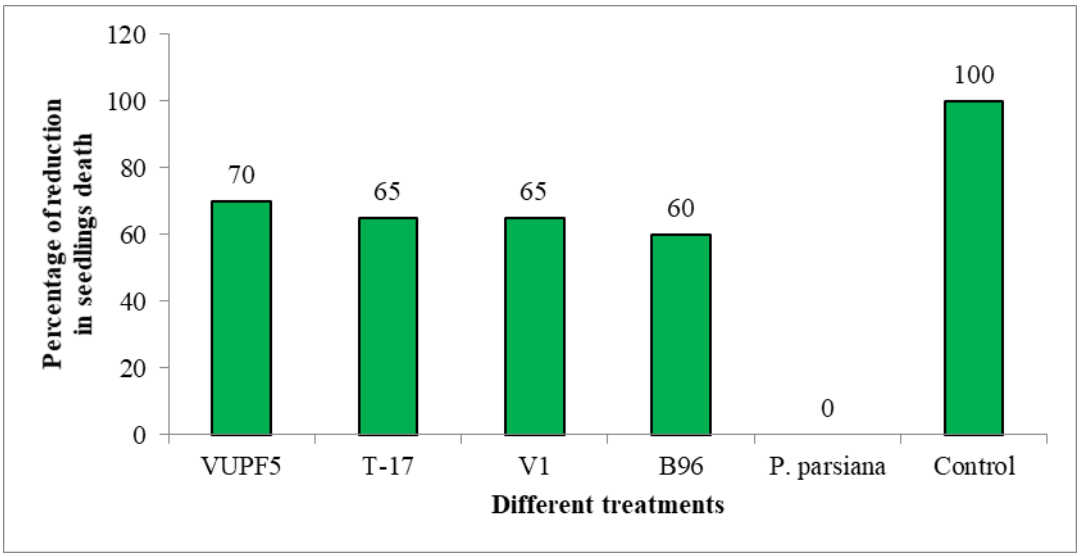

Fig. 3- Percentage of the reduction in seedling death caused by bacterial strains

The use of PGPRs in enhancing plant growth and biological control has attracted the attention of many researchers. In many PGPRs, the production of some metabolites, such as antibiotics, hydrogen cyanide, and siderophores is considered the primary biocontrol mechanism [7]. During the past few years, remarkable advances have been made to understand the molecular and biochemical basis of disease control by some PGPRs. Rhizobacteria employ varied mechanisms. In examining PGPRs, there are at least three steps, including the study of isolates by laboratory tests, verification in pots, and verification under field conditions [17].

The main objective of this study was to identify the most effective strain under laboratory and greenhouse conditions in controlling pistachio gummosis. Saberi-Riseh *et al* [10] studied the ability of 307 isolates from pistachio rhizosphere to control *Phytophthora citrophthora*. Accordingly, 21 gram positive isolates and 10 isolates of fluorescent *Pseudomonads*

had antagonistic effects against *P. citrophthora*. Zhang *et al* [18] reported that the use of PGPR strains, separately or in combination, suppressed squash *Phytophthora* blight under greenhouse conditions. In the current study, bacterial strains, in the zone of inhibition test, prevented mycelial growth of *P. parsiana* at different levels. In terms of antimicrobial activity, the zone of inhibition test is faster and more inexpensive than other laboratory tests. Using this test, a number of samples can be examined quickly for their antimicrobial properties. However, this test does not necessarily indicate that microorganisms are killed by an antimicrobial compound, yet it just implies that they are prevented from growing.

In recent years, the study of volatile organic compounds (VOCs) has been an interesting aspect of research due to their antimicrobial potentials. Accordingly, their antifungal property has turned them into an attractive biocontrol agent in agriculture. In the present study, the bacterial strains were capable of inhibiting the growth of *P. parsiana* by producing antifungal volatile compounds. It has been reported that the release of VOCs by some soil microbes displays anti-microbial activity, induces systemic resistance in crops, and promotes plant growth [19- 20]. Kaddes *et al* [21] examined the effects of two VOCs, namely methyl propanoate (MP) and methyl prop-2-enoate (MA), on barley pathogens. Under different experimental conditions, both compounds exhibited significant antifungal effects. He *et al* [22] evaluated the effects of VOCs produced by *Bacillus methylotrophicus* BCN2 and *Bacillus thuringiensis* BCN10 against five postharvest pathogens of the loquat fruit. Accordingly, the VOCs released by these strains suppressed mycelial growth of all targeted pathogens and disease occurrence. HCN is a volatile compound that could be toxic to plant pathogens [23]. A hypothesis has recently expressed that the function of HCN is associated with its interference in the release of elements, like phosphorus, from mineral substrates [24]. In both antifungal VOC and HCN tests, the VUPF5 strain was at the first place, with T-17, V1, and B96 having followed it in antifungal effects. Thus, it can be concluded that HCN is one of the major components of VOCs.

Similar to laboratory tests in greenhouse experiments, *Pseudomonas fluorescens* VUPF5 delivered the best performance in controlling *P. parsiana*. Besides, the bacterial strains showed high efficacy in improving growth factors. Most especially, strain B96 was quite effective in increasing fresh and dry shoot and root weight, despite yielding weaker laboratory results than other strains. It should be noted that in petri plate assays used for examining biocontrol activity against common plant pathogens, some strains

may be overlooked with this approach. Although all PGPR strains are not cultivable and there are strains not yielding satisfying results at early stages, they can enhance subsequent growth [25]. Upon an increase in the bacterial population, a rise was observed in the fresh and dry shoot and root weight. After introducing rhizobacteria into the soil, they should be able to colonize the rhizosphere so rapidly as to compete with indigenous microflora and exert their beneficial effects. The application of a large initial population of rihzobacteria is one of the factors increasing root colonization. Similar to our results, Yan et al [26] reported that inoculation of plant seeds with different initial populations of bacteria exerted direct effects on the amount of the bacterial population in the roots of tomatoes. Besides, an increase in the initial population inoculated into the seeds resulted in higher colonization of the roots by the bacteria. These strains exerted multiple beneficial effects in the host plants and significantly reduced gummosis incidence. Thus, they could be used in developing new, effective, and safe fungicides, as an alternative to chemicals. Further studies should be carried out on such efficient PGPR isolates with an emphasis on improving biocontrol activity of PGPRs though transferring desirable genes into the strains.

5. Conclusions

According to the results, the bacterial strains had positive effects on controlling *Phytophthora* root and crown rot of pistachio trees under laboratory and greenhouse conditions. Among the tested bacterial strains, *Pseudomonas fluorescens* was the most effective one in controlling the disease. More studies should be done on the efficiency of these bacteria under field conditions to control pistachio gummosis.

Conflict of Interest

The authors of present researches declare that there is no conflict of interest.

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