Biological control of root-knot nematode Meloidogyne incognita in pistachio using bacterial biocontrol agents

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Abstract

Background: Root-knot nematode damage is one of the major challenges in Iranian pistachio production. In the current research, the potential of five isolates of *Bacillus subtilis* (B96, B95, B84, B48, B67) and *Pseudomonas fluorescens* VUPf52 were evaluated to manage root-knot nematode (RKN) *Meloidogyne incognita* in pistachio.

Materials and Methods: The experiment was conducted in a randomized complete block design with three replicates and different treatments depends on the assay.

Results: *In vitro* experiments indicated that all *B. subtilis* treatments could kill second-stage juveniles with 100%, 63.80%, 63.33%, 87.14%, and 78.09% mortality at 72h, respectively. Under *in vivo* conditions, B96 and B95 were able to increase their population in the nematode infected soil more than other treatments. Compared to the control, these isolates also induced a significant decrease in the galling indices of nematodes, egg masses, and adult females per 0.5 gr of pistachio roots. Moreover, the results showed the significant effect of some treatments on the growth factors of pistachio plants. Bacterial suspension of B96 and *P. fluorescens* VUPf52 was applied as a soil drench under field conditions. Field assessments indicated that *P. fluorescens* VUPf52 was not significantly different compared with the negative control; although, B96 decreased the populations of second-stage juveniles (J2) the most.

Conclusion: It is concluded that *B. subtilis* products have the potential to be one of the components in the integrated RKN management in pistachio. Bacterial efficacy on *M. incognita* in pistachio orchards is reported here for the first time.

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1. Introduction

As an essential nutritional and economic commodity, pistachio (*Pistachio vera* L., Anacardiaceae) orchards came from Asia Minor's arid and wilderness regions and are called in Iran as "Green Gold." As the largest producer of pistachio globally, with about 346000 ha of cultivated land, Iran produces around 315000 tons of yield annually [1]. Among the plant-pathogenic nematodes, RKNs (*Meloidogyne* spp.) with an approximated US$100 billion cost annually are one of the most destroying varieties in the world [2]. Agricultural pests constitute ten of the 98 reported *Meloidogyne* species, while five cyst species and six lesion species are economically significant. RKNs are obliged plant parasites observed worldwide. The most harmful species are the *Meloidogyne arenaria, M. javanica., M. incognita*, and *M. hapla* [3]. *M. incognita* and *M. javanica* are the most prominent economic ones in Iranian pistachio orchards [4]. Nematode disease management is very complicated due to inhabiting the soil and damaging the underground sections of plants [5]. Various approaches can be employed to manage *Meloidogyne* spp, for instance, chemical nematicides that are easily applied and more efficient. The negative side effects of chemical nematicides on human and environment are driving calls to decrease their application in the control of *Meloidogyne spp*. Consequently, alternative control methods are required, particularly those environmentally friendly. By using biocontrol agents, *Meloidogyne spp*. can be controlled [6]. In the natural soil ecosystem, bacteria and fungi are among the most prominent soil-borne microorganisms which have proven to be effective BCAs for RKNs [7&8]. Trichoderma is one of the soil microorganisms and possible nematode biocontrol agents in numerous vegetables and crops [9, 10]. *Trichoderma* spp and *Bacillus megaterium* are usual soil bio-agents that manage RKN [11, 12]. Huang et al. [13] reported that a composition of *Syncephalastrum racemocum* and *Paecilomyces lilacinumat* decreased 70% of egg hatching at 50% against *M. incognita* compared to the control. Furthermore, the inoculation with *S. racemonsum*, at 50%, revealed the most larvicidal activity, with the mortality rate reaching a peak of 96.7%. *Pochonia chlamydosporia* var. *chlamydosporia* isolates showed capacity as BCAs for *M. javanica* in pistachio plants [14]. *Bacillus spp*. act as an important agent in controlling *Meloidogyne spp*. by producing lytic enzymes, cyclic lipopeptides [11, 12], and plenty of secondary metabolites. Chitinase and glucanase are part of many lytic enzymes produces by *B. cereus* [15]. The accumulation of population and number of galls were reduced when *Meloidogyne spp* were treated with *Pseudomonas aeruginosa*, *B. subtilis*, and antagonistic fungus *P. lilacinus* [16]. Rhizobacteria compete with the microorganisms in the rhizosphere for colonizing the root system. [17]. Particular root-associated strains of *P. fluorescens* play a restrictive role on soil-born plant pathogens by producing metabolites [18]. In a research to assess Iranian strains of *Pseudomonas* spp. for managing *Meloidogyne incognita* on pistachio orchards, the number of gall formation diminished when treated with the bacteria. Moreover, depending on the exposure time and

bacterial concentration, *M. incognita* juvenile's survival was affected under *in vitro* conditions [19]. *P. fluorescens* VUPf52, *P. fluorescens* VUPf5, *B. subtilis* PRC95, and *B. subtilis* PRC96 were shown to have a notable influence on manifolding indices of *M. incognita* on pistachio seedlings of Sarakhs and Badami cultivar. After four months of treating the seedlings of both cultivars with *M. incognita* and antagonistic bacteria, the number of J2, galls and egg masses reduced in comparison to non-treated seedlings [8]. This research was accomplished to assess the antagonistic and suppression impact of *P. fluorescens* VUPf52 and *B. subtilis* isolates on the activity of second stage juveniles (J2) of *M. incognita* under *in vitro* conditions, examine the growth indices of pistachio plants and the suppressive effects of the promising bacterial isolates under in vivo conditions, and evaluate their efficacy to increase plant growth using soil drench application. This research reports the PGPRs (Plant Growth Promoting Rhizobacteria) effectiveness on *M. incognita* in pistachio orchards for the first time.

2. Materials and Methods

Bacterial isolates used in the experiment

B. subtilis isolates (B96, B95, B48, B84, B67) were obtained from the culture collection unit at the Iranian Pistachio Research Institute, Rafsanjan, Iran, and *P. fluorescens* VUPf52 were retrieved from the culture collection unit at the Vali-e-Asr University of Rafsanjan [19] (All of these strains were previously isolated from pistachio rhizosphere in several regions of Rafsanjan). Bacterial isolates were stored at -80℃ in 15% glycerol and Luria Broth (LB) to be used in further studies. They were identified based on biochemical and physiological characterizations [20] and the molecular method described by Wang et al. [21].

Preparation of antibiotic-resistant mutants

In order to track the desired isolates, antibiotic-resistant mutants were prepared. Tetracycline and chloramphenicol were used for this purpose. First, a single colony from a fresh cultural medium of each bacterium was gradually added to concentrations of 10, 20, 35, 50, 70, 100, and 150 ppm of tetracycline. Then, the colony with the ability to grow at 150 ppm of this antibiotic was selected and, after stabilization in this concentration, were entered into the same concentrations of chloramphenicol. In order to stabilize the desired mutant, the colony with the ability to grow in the highest concentration of chloramphenicol was cultured on a medium containing 150 ppm of tetracycline. After ensuring growth, antibiotic-resistant mutants were cultured again on NA (Nutrient Agar) and stored at −80℃. These mutations were used under field conditions.

Sampling and nematode extraction

Root and soil samples were taken from the Badami-Zarandi cultivar pistachio orchard in Rafsanjan. They were put in bags and transferred to the lab for future examinations. Pure cultures of egg masses were kept on tomato plants (*Lycopersicon esculetom* cv. Rutgers) grown in pots for four months to distinguish the large-scale inoculum production of *Meloidogyne*. To diagnose the *Meloidogyne* spp., single root galls (n= 10-20) were cut. Adult females were discarded and utilized to prepare the perineal patterns for identifying them based on species [22]. The perineal patterns matched to *M. incognita* as detailed observation of 10-20

female nematodes indicated [23]. Egg masses were separated from the roots with forceps, washed with sterile water, placed in sodium hypochlorite solution (0.5%), agitated for three min, and washed with sterile water on a sieve (500 mesh, aperture = 0.025) to prepare second-stage juveniles [19]. The egg suspension was passed through a small coarse sieve (500 mesh) coated with tissue paper in a Petri plate (18 ×2.5 cm, diameter × height, ca. 70 egg masses per dish), which contained an adequate amount of water. Second-stage juveniles were gathered in sterile water after incubation at 27± 5℃ for two days. The suspension containing nematode juveniles was sanitized for 15-20 min with tetracycline (5mg⁄L) and streptomycin sulfate (0.044g⁄L). then washing three times with dsH_2O. Pistachio seedlings (Badami-Zarandi cultivar) were grown in sterilized soil (1000gr) and injected with the suspension, containing 2000 second-stage juveniles in each pot, for *in vivo* studies.

In vitro suppressive impact of *B. subtilis* isolates on juvenile's mortality of *Meloidogyne*

In order to obtain fresh culture for the petri plate assay, *B. subtillis* isolates were grown on NA at 25℃ for 24 h. Subsequently, they were suspended in dsH_2O. The culture absorbance was monitored with a UV light absorbance (Spectronic®, UK) using a wavelength of 450nm until reaching an absorbance of 0.5 (equivalent to 10^{10} CFU ⁄ ml). The impact of bacterial isolates on juvenile's mortality of *M. incognita* was investigated by adding 1ml of water containing 70 freshly sterile juveniles of *M. incognita* to each Petri dish (6 cm diameter), followed by 1ml of the bacterial suspension. The plates were incubated at 26-27 ℃. All treatments involved three replicates, and experiments were repeated two times. For the control treatment, *M. incognita* juveniles were treated with a culture medium in the same way. Mortality was noted after 24, 48, and 72 h and was examined by jabbing the juveniles with a sterile needle. After 72h, the juveniles were transferred to distilled water (24h) to ensure that no recovery appeared [24].

In vivo activity of *B. subtilis* isolates against *Meloidogyne* juveniles

Pistachio seeds (cv, Badami-Zarandi) were disinfected in 1% NaOCL for 4 min, washed with sterile distilled water, and soaked in 2 gr⁄L iprodione+carbendazim, followed by soaking overnight in sterilized water and pre germination on a moist filter paper in Petri dishes. Each pot (25 cm in diameter) was containing one seedling and sterilized soil (sand and perlite; 1:2 V⁄V, 1000gr). When pistachio seedlings reached the 6-8 leaves stage, 10 ml of bacterial suspensions were injected into a hole around each plant (10^{10} CFU ⁄ ml for each pot). Simultaneously, approximately 2000 juveniles of *M. incognita* in 20 ml sterile distilled water, were inoculated for each pot. The suspension was spilled into a shallow trench build around the root tips of each test plant [25] and covered immediately with topsoil [26]. Pots were kept at 24 ℃± 2℃ in a complete randomized design with three replicates containing one seedling. Three months after nematode inoculation, pistachio plants were harvested. Roots were rinsed in water and smudged lightly dry; further, the fresh weights and heights of the pistachio shoots and roots were recorded. Moreover, the reproduction factor and the number of galls, egg masses, and *M. incognita* juveniles in the soil were determined.

Field studies

To assess the effectiveness of antagonistic bacteria under field conditions, antibiotic-resistant mutants of B96 and *P. fluorescens* VUPf52 were used in this study. B96 was used owing to the promising results obtained under *in vitro* and *in vivo* conditions. Additionally, *P. fluorescens* VUPf52 was, in our previous studies, illustrated to be effective in the suppression of RKN, *M. incognita* of pistachio under *in vitro* and *in vivo* conditions [20]; this strain was preferably applied in the field condition to compare with *B. subtilis* in current research. Field studies were accomplished in a naturally *M. incognita* infested pistachio field located in Safaieh village, Rafsanjan, Iran. Initially, the soil density of *M. incognita* was indicated by taking random soil cores (from 30-60 cm depth at different sites). Then, the experiments were conducted in a factorial randomized complete block design with ten trees as replicates per each treatment. Bacterial isolates were re-suspended in sterile distilled water, and the concentration was adjusted to give 10^{10}cells/ml. Each bacterial isolate was used in one dose, 20 ml of the bacterium suspension (each contained 10^{10} bacterium cells and then diluted in a liter of water), at a rate of one L/m^2 as a soil drench application. Soil sampling was done at intervals of one day before the study, one week, two months, and four months after treatment for two consecutive years. Each soil sample comprised three sub-samples randomly extracted from a tree rhizosphere (30-60 cm depth) and mixed into a single sample showing one tree. The second-stage juveniles (J2) (per 200 gr soil) were taken from the soil by the decanting and sieving technique [26] and then counted. Sterile distilled water was used for the positive control and *M. incognita* as the negative control.

The bacterial population was ascertained in the rhizosphere of pistachio seedlings after nematode inoculation. Shortly, one gram of soil was transported to one ml of sterile distilled water and then shaken completely. For each sample, a serial dilution was prepared and spread on NA medium in three replicates. The bacterial colony was conducted after incubating at 28 ℃ 24 h under dark conditions. In order to detect antibiotic-resistant bacteria, a suspension containing nematode juveniles was sterilized with tetracycline (5 mg/L) for 30 min and then rinsed three times with sterile distilled water and cultured on NA plus antibiotic. This experiment was repeated for two years, and the recorded data were the average of data obtained from both years.

Statistical analysis

The average values of juvenile mortality, the population of bacterial strains, *M. incognita* juveniles in soil (100-200gr), the number of galls per gram of root, fresh and dry weight, and heights of pistachio shoots and roots were separately determined for each replication. The data were analyzed using the Proc GLM procedure (SAS Release Version 9.1. SAS Institute, Cary, NC) by one-way analysis of variance (ANOVA). Mean comparison was made using Duncan's multiple range test (AVOVA, $P>0.05$, SAS version 6.12).

3. Results

In vitro experiment

According to Table 1, all tested bacterial isolates significantly increased the juvenile (J2) mortality percentage compared to the untreated control. All treatments exhibited a high capacity of mortality after 72h. Nevertheless, after 24h,

B96 showed a particular impact, obtaining 85% juvenile mortality. Throughout two first exposure times, the least effective isolate was B67, with 5.71% juvenile mortality after 24h and 14.28% after 48h. B96 had the best impact on nematode after 24h, 48h, and 72h, resulting in juvenile mortality of 85.23%, 98.09%, and 100%, respectively.

Table 1. In vitro efficacy of *Bacillus subtilis* isolates on juvenile's mortality of *Meloidogyne incognita* after 24, 48, and 72 h

Treatments	Mortality rate		
	24h	48h	72h
B96	85.23^{a}	98.09^{a}	100^{a}
B95	12.38^{bc}	21.90^{b}	63.8^{c}
B48	17.14^{b}	37.14^{b}	87.14^{b}
B84	5.23^{c}	20.95^{b}	63.33^{c}
B67	5.71^{c}	14.28^{b}	78.09^{b}
Control	0	0	0

Within columns, mean values followed by different letters are significantly different ($P \leq 0.05$).

In vivo experiments

Assessment of *B. subtilis* population in the rhizosphere

All bacterial isolates survived until the end of the experiment. The population of B96 and B95 isolates in soil increased more than others. These isolates had a more rhizosphere competence around pistachio roots (Figure 1). The population of B48, B84, and B67 also significantly increased. After inoculation with nematodes, B96 had the highest population, although B96 did not show a significant difference with B95, B48, B84, and B67 treatments (Figure 1). Nevertheless, it does not indicate that their efficiency in controlling nematodes is changed.

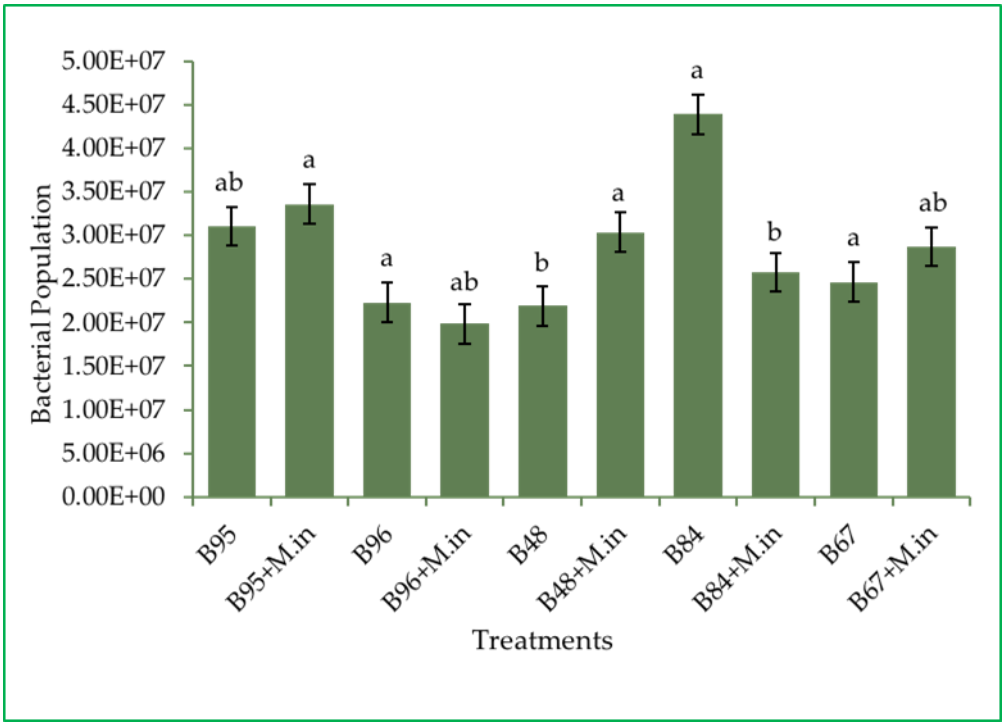

Figure 1. **Bacterial population in the rhizosphere**; Within columns, mean values followed by different letters are significantly different (P≤ 0.05); B (*Bacillus subtillis*), M. in (*Meloidogyne incognita*).

Effect of *B. subtilis* isolates on nematode population

Under *in vivo* conditions, *M. incognita* was inoculated on pistachio plants; however, in the absence of bacterial isolates, the most significant number of galls (238.25) were observed on their roots (Table 2). Nevertheless, their quantity was reduced remarkably in the plants treated with bacterial isolates. B96 reduced the number of galls the most compared to the negative control. Moreover, the number of the nematode J2 in the soil decreased notably in comparison to negative control, when treated with B96 and B95. In the case of second-stage juveniles in the soil, B96 was the most effective isolate. All isolates, except B84 and B67, also remarkably reduced the number of nematode egg masses in the soil compared to the untreated control. In this case, B96 had the best effect on lowering egg masses. Nematode reproduction was notably decreased in plants treated with B96 and B95. Generally, B96 showed a high percentage of *M. incognita* control under *in vivo* conditions, followed by B95, B48, B84, and B67 with 68.43%, 59.20%, 52.15%, 46.69%, and 46.52%, respectively (Table 2).

Table 2. The efficacy of *B. subtilis* isolates on *M. incognita* population under *in vivo* conditions

Treatments	Number of J2 (100gr)	Number of galls	Egg masses	Reproduction factor	Control %
M.in	13.52^{b}	238.25^{a}	47.25^{a}	0.15^{a}	-
M.in +B96	23.32^{a}	75.2^{d}	20.2^{b}	0.083^{b}	68.43
M.in +B95	19.28^{a}	97.2^{cd}	22.8^{b}	0.1^{b}	59.20
M.in +B48	12.4b	114^{bc}	26^{b}	0.16^{a}	52.15
M.in +B84	12.85^{b}	127.8^{b}	36.2^{ab}	0.16^{a}	46.69
M.in +B67	10.99^{b}	127.4^{b}	37.2^{ab}	0.18^{a}	46.52

Within columns, mean values followed by different letters are significantly different ($P \leq \mathbf{0.05}$)

Impact of *B. subtilis* isolates on plant growth indices under greenhouse conditions

Plant growth indices decreased in treatments with nematodes compared to the nematode-free control (Figures 2 and 3). In the case of shoot length, there was no difference between treatments and untreated inoculated plants [data are not shown]. However, the assessment of dry and fresh weight of both shoots and roots indicated that B96 and B95 stimulates the growth of both shoots and roots of pistachio plants in nematode infected soil. B84 application gave the highest fresh and dry shoot weight, followed by B96 and B96 + *M. incognita* (Figure 2). In the case of fresh and dry root weight, B95, B96, B48, and B95 + *M. incognita* gave the best results (Figure 3). The control treatment displayed the least shoot and root weight. Thus, the maximum plant growth was obtained by B95 + *M. incognita*, while the minimum was in controls (Figures 2 and 3).

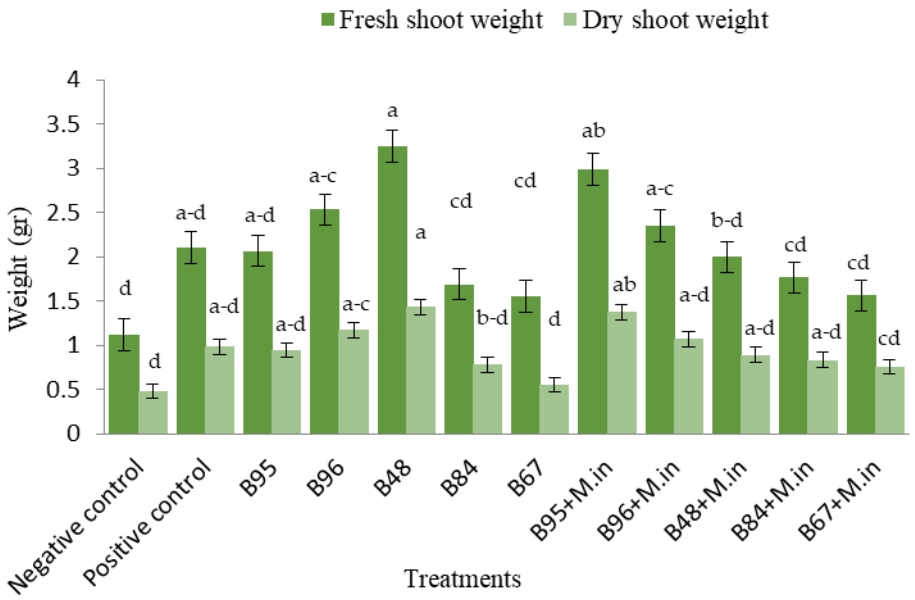

Figure 2. The efficacy of different treatments on shoots fresh and dry weights in pistachio plants; Within columns, mean values followed by different letters are significantly different (P≤ 0.05); B = *Bacillus subtilis*, M. in = *Meloidogyne incognita.*

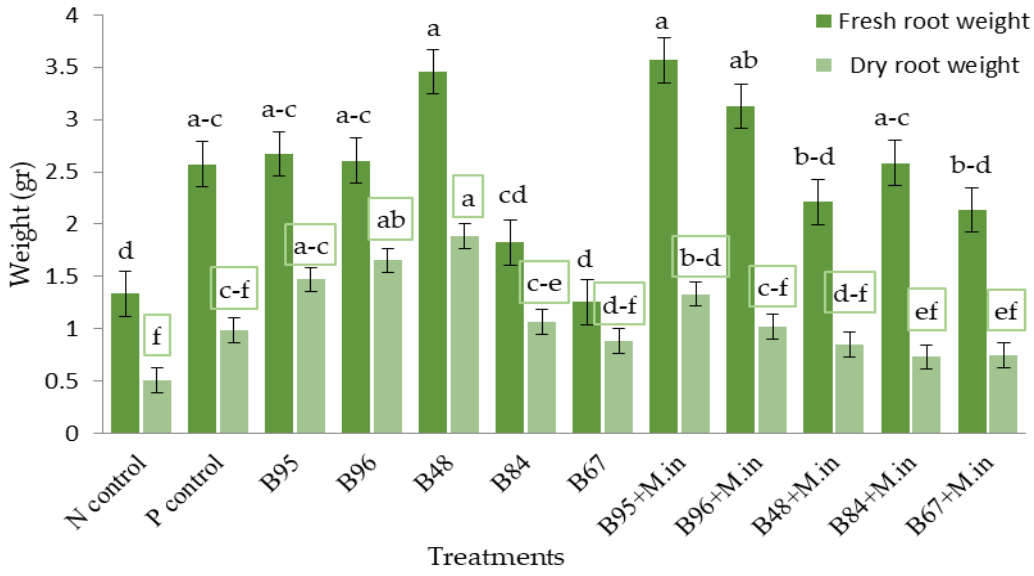

Figure 3. The efficacy of different treatments on roots fresh and dry weights in pistachio plants; Within columns, mean values followed by different letters are significantly different (P≤ 0.05)

Field experiments

The *B. subtilis* B96 and *P. fluorescens* VUPf52 decreased RKN, *M. incognita*, in pistachio field under natural field infestation for two years. The evaluation was done based on the number of J2 in the soil at different times. As observed in the first sampling time, the treatments were not significantly different. After one week of inoculation, *P. fluorescens* VUPf52 had better results in decreasing the number of second-stage juveniles in the soil. No difference was observed between *B. subtilis* B96 and the positive control. In the third and fourth sampling time, the lowest number of J2 was achieved when *B. subtilis* B96 was applied (Figure 4).

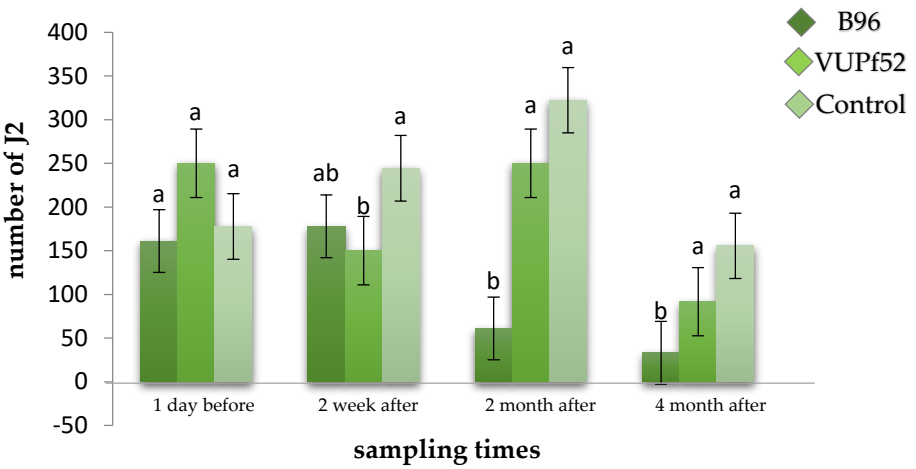

Figure 4. Root-knot nematode juvenile number (J2) recovered from soil; Mean values followed by different letters are significantly different (P≤ 0.05)); Data are the average of data obtained in 2 consecutive years.

4. Discussion

This experiment confirmed the PGPRs' ability against the RKNs as biological control agents. The outcomes revealed that these bacterial isolates are potential enough in the biological control of *M. incognita*, confirming previous findings [27-28-29] that reported the biocontrol capability of some antagonistic bacteria against plant-parasitic nematodes, including *Meloidogyne* spp. Various researches have indicated the biocontrol potential of *P. fluorescens* and *B. subtilis* against numerous plant pathogens. [30-31]. In this research, the bacterial isolates provided a remarkable impact on protecting amplification indices of *M. incognita* in pistachio plants of the Badami-Zarandi cultivar. Moreover, the fresh and dry weights of shoots and roots were enhanced by bacterial isolates in the absence of the nematode. The positive outcomes retrieved under the *in vitro* trial showed that *B. subtilis* isolates had strong nematicidal activity. While all treatments showed larvicidal activity, B96 had the most efficacy. These results are in line with [7], in which *B. subtilis* B10 was repotted to kill 90.7% of J2 of *M. incognita* at 50% concentration. *B. subtilis* Rh-18 showed the greatest antagonistic

activity against *Pratylenchus loosi* with an 86.01% mortality rate [32]. Under the *in vivo* condition, treatment with B96 and B95 induced and stimulated the growth of the pistachio plants effectively compared with the others. The *in vivo* assay in the current experiment confirmed previous achievements by Basyony and Abo-Zaid [7] on tomato plants, reporting the growth enhancement of nematode-infected plants by 33%-72% using *B. subtilis* B10. According to another study, shoots' dry weights showed the greatest increase, reaching 57.65% over control when *B. subtilis* was applied as a soil drench [7]. B96 and B95 decreased galls number, egg masses, reproductive factors, and second-stage juveniles' number in the root of pistachio plants. Based on Fakhreldin [33], the number of galls decreased by 57.92% when *B. subtilis* isolates were applied to tomato plants against *M. javanica*. The present research results supported the findings of Basyony and Abo-Zaid [7], who concluded that the number of egg masses and galls of *M. incognita* in the soil were reduced by *B. subtilis* isolates. According to Zeynadini-Riseh et al. [8], *B. subtilis* induced a great decrease in nematode multiplication. The instability and variability of root colonization are often limiting factors in using rhizosphere by antagonistic bacteria. In the absence of *M. incognita*, the population of B96 and B95 increased in comparison to other treatments. In the presence of nematode, only B96 had a significant establishment in the soil and rhizosphere of pistachio plants. This could be related to its colonization ability since one important reason for the variation in bacteria's antagonistic behavior is the difference in colonization rate at various times and places. The instability or variability of root colonization is often a limiting factor in using rhizobacteria [34]. This was the first experiment on biocontrol of RKNs in pistachio orchards by *P. fluoresces* and *B. subtilis* under field conditions. As observed in the results, as expected, the evaluation of the samples one day before the inoculation indicated treatments not to be significantly different in reducing the number of J2. However, after one week of inoculation, *P. fluoresces* VUPf52 caused better juvenile mortality than B96 and the untreated control, indicating the Pseudomonas bacteria's success in favorable moisture conditions. Fluorescent pseudomonads have many features, making them one of the most potential PGPRs which have received much attention in recent years as biocontrol agent. Fluorescent pseudomonads are known for the production of diverse microbial metabolites, the potential for rapid colonization, the ability to utilize seed and root exudates, the adaptation to biotic and abiotic stresses and the ability to proliferate under *in vitro* and *in vivo* conditions [35]. In the third and fourth sampling times, B96 showed more reduction in the number of J2 in the soil, indicating more effects on nematode control. After four months, a quite large decrease was observed in all treatments, attributed to some natural fluctuation in the nematode life cycle due to the cold season. Bacillus, as a large group of bacteria, has recorded diversified effects on plant-parasitic nematodes. *Bacillus* spp. with nematicidal impacts include *Bacillus subtilis* [36]. Many previous studies have shown *Bacillus subtilis* ability as a bio-agent to control soil-borne diseases and plant-parasitic nematodes. Beneduzi et al. [37] indicated the effects of *B. subtilis* isolates via local antagonism to nematodes or systemic resistance induction against pathogens. Another prominent feature of these bacteria is the production of endospores, allowing their use in biological control because of enduring harsh environmental conditions.

Therefore, compared to *P. fluoresces* VUPf52, B96 possibly had a better effect on the root-knot control due to endospore production. In southern Iran, *Meloidogyne spp.* create at least five generations on trees annually [38]. Based on our results, these bacterial strains are potential bio-stimulants for sustainable crop protection to manage *M. incognita* on pistachio orchards. Further investigation is needs on the effect of these agents combined with each other or with other antagonistic fungi, and bacteria under *in situ* conditions.

5. Conclusion

Application of biocontrol agents for managing RKNs has been ongoing for decades. So, it is crucial to continue improving these methods and making them more efficient. In this research, *B. subtilis* isolates were potential in controlling RKN caused by *M. incognita*. Therefore, future studies should be expanded in terms of determining the mechanisms of antagonistic activity of B96 and a formulation to show the best results.

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