

Formulation Development of Some Fluorescent *Pseudomonads* for Controlling of *Meloidogyne incognita* on Pistachio

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ABSTRACT

Plant growth which promotes rhizobacteria (PGPR) has a potential role in controlling root-knot disease. To reach the aim of this study, we used rhizobacteria replaced by chemicals to reduce this problem in Pistachio trees. Based on the previous studies, some strains with high ability were selected to reduce root knot severity. Three different formulations were prepared, using different carriers of materials named; wheat bran, dried apple and talc powder. we have used six individual strains of *P. fluorescens* and one combination of two strains (Vupf506+Vupf52). These formulations were tested to control *M. incognita* in Pistachio seedlings in the greenhouse condition. The wheat bran based treatments was effective in reducing disease by 32.7 to 88.9%. The bacteria survived longer in wheat bran-based and talc-based formulations than in dried apple-based formulation. The results of the study indicated that organic carriers can be efficiently used to improve the stability and efficiency of biocontrol-active microorganisms in controlling the Pistachio root-knot disease.

keywords: Biological control, *Meloidogyne incognita*, *Pistacia vera*, *Pseudomonas fluorescens*.

INTRODUCTION

Management of soil-borne pathogens seems always problematic (Haware and Kannaiyan 1992). Soil solarization, crop rotation and mixed cropping are the most practical methods to reduce soil borne pathogens (Sullivan, 2004). In the case of nematodes, nematicides can be used to reduce damage on the field condition. However, their use brings about expenses as well as being environmentally undesirable (Song and Goodman, 2001). Biocontrol can be an eco-friendly way of managing root-knot nematode, offering an alternative to nematicides (Prasad and Rangeshwaran, 2000). The previous studies on *M. incognita* showed that diseases could be controlled by the application of *Pseudomonas fluorescens* as a soil treatment (Maurhofer *et al.*, 1994). *Pseudomonas* spp. is the most extensively studied plant growth promoting rhizobacteria

(PGPR). Plant pathogenic agents are discovered to be more resistant against synthetic chemicals. Thus, the development of bio-formulations is replaced by nematicides for the management of plant disease or used in integrated control appears desirable (Ardakani *et al.*, 2010). The major constraint to use biological control extensively on ground condition is the absence of knowledge about mass-production and proper delivery of biocontrol agents (Papavizas, 1985). Commercial application of *P. fluorescens* to control soil-borne diseases depends upon the development of formulations in which the bacteria can survive for a considerable period of time (Vidhyasekaran and Muthamilan, 1995). Several attempts were made to prepare formulations of *P. fluorescens*. As a result, peat-based (Hagedorn *et al.*, 1993), talc-based (Hofte *et al.*,

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1994), bentonite-based and rice-bran based formulations (Ardakani *et al.*, 2010) of specific strains of *P. fluorescens* were introduced. The efficacy of carriers can affect antagonistic ability. It should be noted that bacteria in the environment must be stabilized to allow them to show their biocontrol abilities.

The impact of various carrier materials to support the growth of *P. fluorescens* vital period was investigated at storage (Meena, 2010). Talc based formulation is considered to be the most widely used carrier and has been reported to manage several crop diseases in India (Samiyappan *et al.*, 1988. According to the previous studies by the authors, four of 11 native strains of *P. fluorescens* were found to provide significant control of root knot nematode in pistachio seedlings, CHA0 and CHA89 as well-known strain for biocontrol activities use. Talc and other two new natural substrates, wheat bran and dried apple powder formulations, were tested as carriers. To the authors' knowledge, there is insufficient information about *Pseudomonas* spp.

effect on Pistachio root-knot nematodes disease. The use of PGPR strains as biocontrol agents against root-knot nematode in the two new formulations (wheat-bran and dried apple powder as carriers fixed uniform bacterial population and provides slow release of bacteria for a long period.

Materials and Methods

Preparation of mineral and organic carriers. Three powdered carriers such as, talc, dried apple, and wheat bran were chosen (Table 1). To prepare apple powder, unripe apple fruits *Malus domestica* var. *antonovka* were surface sterilized with 1% NaOCl for 5 min, rinsed three times with sterilized water and oven dried at 65°C for 10 days. Once dehydrated completely, the apples pulverized in a conventional electric grinder. All carriers were steamed sterilized at 140 KPa for 30 min, and dried aseptically in glass trays for 12 h at 50°C. Each carrier materials were kept in storage condition of 28 to 33 °C on a laboratory bench for testing greenhouse studies and survival activities.

Table 1. Bioformulations used in this study.

carrier/ Treatments	Ingredients/Methods	Strains/s
Talcum powder (A)	Suspension of <i>P. fluorescens</i> strain (400ml) containing 1×10^{12} CFU ml ⁻¹ mixed with talcum powder (100g) and CMC (25g).	VUPf52, VUPf5, VUPf205, VUPf49, CHA0, CHA89
Dried Apple Powder (B)	Suspension of <i>P. fluorescens</i> strain (400ml) containing 1×10^{12} CFU ml ⁻¹ mixed with dried apple powder (50g), talcum powder (50g) and CMC (10g).	VUPf52, VUPf5, VUPf205, VUPf49, CHA0, CHA89
Wheat Bran (C)	Suspension of <i>P. fluorescens</i> strain (400ml) containing 1×10^{12} CFU ml ⁻¹ mixed with wheat bran (50g), talcum powder (50g) and CMC (25g).	VUPf52, VUPf5, VUPf205, VUPf49, CHA0, CHA89
2+A	Suspension of <i>P. fluorescens</i> strains (400ml) containing 1×10^6 CFU ml ⁻¹ of each strain mixed with talcum powder (100g) and CMC (25g).	VUPf506+VUPf52
2+B	Suspension of <i>P. fluorescens</i> strains (400ml) containing 1×10^6 CFU ml ⁻¹ of each strain mixed with dried apple powder (50g), talcum powder (50g) and CMC (10g).	VUPf506+VUPf52
2+C	Suspension of <i>P. fluorescens</i> strains (400ml) containing 1×10^6 CFU ml ⁻¹ of each strain mixed with wheat bran (50g), talcum powder (50g) and CMC (25g).	VUPf506+VUPf52

Experimental procedures

Preparation of bacterial suspension:

Pseudomonas fluorescens strains were provided by the Vali-E-Asr University, Biocontrol Collection, Department of Plant Protection, Rafsanjan, Iran. Based on the results of the previous studies (Khatamidoost *et al.*, 2014), seven treatments were chosen. Four native isolates with highly disease reduction (VUPf52, VUPf5, VUPf205, VUPf49) plus CHA0 and CHA89 (the global strains to compete with native ones) and, the mixture of two strains (VUPf506 because of its ability in increasing growth factors+ VUPf52 because of its high ability in nematode control). The bacterial strains kept at -80°C in 44% glycerol. Finally cells were first grown on Nutrient Agar (NA, Himedia, Mumbai, India) medium to verify their purity. Fresh cultures used for each part of study. The inoculum produced by taking a loopful of the bacterial colony grown for 24 h on NA. The concentrations of bacteria to approximately 1×10^{12} CFU (colony forming units) ml^{-1} as a suspension using spectrophotometer assays (Thompson, 1996).

Development of formulations of Pseudomonas strains:

A concentration of 10^{12} CFU ml^{-1} was used to inoculate talc-based, dried apple powder-based and wheat bran-based formulations. The carriers were packed into a polyethylene bag and autoclaved for 30 min at 121°C on each of two consecutive days. Four hundred milliliters of the bacterial suspension contained 1×10^{12} CFU was added to 100g of each carrier and mixed well under sterile condition. Then, to emulsify, maintain and absorb water, mixture of (CMC) (carboxymethylcellulose) was added to all formulations (1:4 v/v) (Table 1).

Afterwards, they were mixed well under sterile condition (Vidhyasekaran and Muthuamilan, 1995). The pH of the formulation adjusted to 7.0 by adding CaCO_3 . Then, the product was dried with no exposure to sun to reduce the moisture content to less than 20% and packed in polypropylene bags, sealed and incubated at room temperature ($25 \pm 2^\circ\text{C}$). One gram of each formulation was drawn every 30 days for up to 4 months and the viability of the bacterial populations assessed by dilution plating on nutrient agar.

Greenhouse studies:

The efficacy of formulated *P. fluorescens* strains in controlling Pistachio root-knot nematode was evaluated in the greenhouse condition. Seeds of Pistachio (cv. Badami) were labelled as highly susceptible to root-knot nematode (Madani *et al.*, 2012) obtained from the Iranian Pistachio Research Institute, Rafsanjan, Iran. Pistachio seeds without any stains or indications of pests or disease damage were sterilized on the surface with 1% NaOCl for 5 min and rinsed three times with sterile water. Two-month-old seedlings were produced by sowing one seed in each 25 cm diameter pot filled with a sterilized mixture of sand and clay (2:1; v/v). Soil was collected from Pistachio orchards in Rafsanjan and autoclaved for 45 min at 121°C. One gram of bacterial formulation was placed in the pot at the base of the Pistachio seedlings, by removing soil, placing it, and then covering it up. A day after inoculation (Ardakani *et al.*, 2010), seedlings in each pot were inoculated with 10 ml of a suspension containing 2000 freshly hatched second stage juveniles (J2) of *M. incognita* by adding the required amount of inocula through four narrow holes made around

each plant. Plants kept at $24 \pm 2^\circ\text{C}$ in a greenhouse. After 2 months' plants uprooted, galls, and egg masses were counted. The indices gall index, egg mass and reproduction factor were recorded according to Sharma et al. (1994). The reaction of Pistachio seedlings, expressed as the gall and egg mass production, was scored as 0 = no galls, 1 = 1 to 2 galls, 2 = 3 to 10, 3 = 11 to 30, 4 = 31 to 100, and 5 = more than 100 galls (Bridge *et al.*, 1982).

The experiment included 21 treatments and five replicates. Pistachio seedlings which were inoculated with 2000 J2 *M. incognita* but not with *P. fluorescens* were considered as the control group, whereas seedlings neither inoculated with *M. incognita* J2 nor with *P. fluorescens* were considered as the normal group. Sixty days after inoculation with the J2s, plant growth (height and weight) and nematode damage were recorded.

Populations of *P. fluorescens* in the rhizosphere were determined after dilution plating on NA. Briefly, 1 g of root rhizosphere transferred to 1 ml of sterile distilled water and mixed thoroughly. A serial dilution was prepared from the suspension. One hundred microliters of each dilution spread on NA in three replicates. The colonies recorded after 24 h incubations at 28°C in darkness.

Statistical analyses:

All data subjected to analysis of variance (ANOVA) used SAS Release Version 9.1, SAS Institute, Inc., and Cary, NC. Differences between treatments were determined by Fisher's LSD tests at 5% significant level.

Results

The effect of the bio-formulations of bacteria on reduction of disease severity:

Bio-formulations of all native strains of

P. fluorescens in three carrier formulations significantly reduced the severity of producing gall (Table 2) of symptoms caused by *M. incognita* compared with the control group. The formulations of *P. fluorescens* were found to be effective in reducing the Pistachio root-knot nematode gall number in the greenhouse condition when applied as a powder by 88.9% (2+C) to 48.5% (CHA89B) with the exception of CHA89C and CHA0, which did not differ significantly from the control. Table 2 shows that high gall and egg mass reduction are related to 2+ C treatment with the inhibition rate of 88.9%, and with high performance in all factors in growth and disease reduction. In dried apple formulation, the rate of disease reduction ranged from 84.9% with formulation 52B to 2+B (58.3%) under greenhouse condition. In talc formulation, the rate of disease reduction varied from 70.1% to 41.4%, but the effect was not significantly greater than the effect of bran wheat and dried apple formulation treatments.

The most effective of strains in different formulations to reduce disease were VUPf52 strain of wheat bran formulation with 87.0% (52C), apple powder formulation of strain VUPf52 with 84.9% (52B), and VUPf5 strain in talcum powder formulation with 70.1% (52A) of disease control. The bacterial population in the dry weight of rhizosphere observed in treatment 2+C (2.4×10^7).

Survival of P. fluorescens strains in the three carrier formulations. In all carrier formulations, *P. fluorescens* CHA0 survived up to 30 days without considerable decline. Similar results were found with *P. fluorescens* VUPf52. After 60 days of storage about 2.48×10^{10} CFU also detected in wheat bran-based formulation from

initial population 1×10^{12} . Although, subsequently, there was a decline in population, 120 days after incubation the bacterial population was 1.3×10^2 to

8.9×10^6 per gram in dried apple-based and wheat bran-based formulations (Fig 2).

Table 2. Impact of the bioformulations of *Pseudomonas fluorescens* strains on reduction of disease severity.

Treatments	Gall	G.I. ^a	Egg Mass	R.F. ^b	CFU ml ⁻¹	Disease Reduction (%)
Control ^a	54.0 ^a	4.3	59.0 ^a	0.98 ^a	0 ^c	-
52A ^d	18.0 ^{b-e}	3.4	22.0 ^{b-d}	0.14 ^{b-d}	$1.16 \times 10^{7a-c}$	66.6
52B	8.1 ^{de}	2.3	6.8b-d	0.14 ^{b-d}	$8.83 \times 10^{6b-e}$	84.9
52C	7.0 ^{de}	2.6	5.0 ^{cd}	0.33 ^{b-d}	$1.16 \times 10^{7a-c}$	87.0
5A	16.2 ^{b-e}	3.2	22.7 ^{b-d}	0.14 ^{b-d}	$3.09 \times 10^{6c-e}$	70.0
5B	10.5 ^{c-e}	3.0	6.5b-d	0.10 ^{cd}	$7.81 \times 10^{6b-e}$	80.5
5C	13.0 ^{c-e}	3.1	22.7 ^{b-d}	0.07 ^d	$1.54 \times 10^{7a-d}$	75.9
205A	29.0 ^{b-d}	3.6	24.5 ^{b-d}	0.33 ^{b-d}	$8.12 \times 10^{6b-e}$	46.3
205B	13.0 ^{c-e}	3.0	9.3b-d	0.05 ^d	$6.94 \times 10^{6b-e}$	75.9
205C	26.3 ^{b-e}	3.5	23.0 ^{b-d}	0.14 ^{b-d}	$5.45 \times 10^{6b-e}$	51.3
49A	19.5 ^{b-e}	3.3	13.0 ^{b-d}	0.10 ^{cd}	$4.52 \times 10^{6c-e}$	63.9
49B	27.7 ^{b-e}	3.6	27.3 ^{b-d}	0.17 ^{b-d}	2.40×10^{7a}	48.5
49C	19.7 ^{b-e}	3.3	17.7 ^{b-d}	0.05 ^d	2.02×10^{7ab}	63.6
2+A ^e	27.7 ^{b-e}	3.5	28.7 ^{bc}	0.19 ^{b-d}	$7.24 \times 10^{6b-e}$	48.8
2+B	22.5 ^{b-e}	3.4	23.0 ^{b-d}	0.25 ^{b-d}	$4.15 \times 10^{6c-e}$	58.3
2+C	6.0 ^e	2.4	4.0 ^d	0.17 ^{b-d}	2.40×10^{7a}	88.9
CHA0A	31.7 ^{a-c}	4.0	28.0 ^{b-d}	0.16 ^{b-d}	$3.65 \times 10^{6c-e}$	41.3
CHA0B	20.0 ^{b-e}	3.3	17.3 ^{b-d}	0.15 ^{b-d}	$5.15 \times 10^{6b-e}$	62.9
CHA0C	17.3 ^{b-e}	3.2	13.7 ^{b-d}	0.18b-d	$3.03 \times 10^{6c-e}$	67.9
CHA89A	25.0 ^{b-e}	3.5	25.5b-d	0.42 ^{bc}	3.48×10^{5e}	53.7
CHA89B	27.8 ^{b-e}	3.6	24.8 ^{b-d}	0.22 ^{b-d}	1.16×10^{6de}	48.5
CHA89C	36.3 ^{ab}	4.0	31.0 ^b	0.43 ^b	1.14×10^{6de}	32.7

Each value is mean of four replicates; values within a column followed by different alphabets are significantly different from each other at $P \leq 0.05$ according to the Fisher's LSD test. ^aGall index. ^bReproduction factor = Pf/Pi (Final population/Initial population).

^cControl represents seedlings inoculated with *Meloidogyne incognita* 2000 J2 but not with *P. fluorescens*.

^dThree carrier formulations of *P. fluorescens* (A: talcum powder, B: dried apple powder and C: wheat bran). ^eMixture of two strains (VUPf52 and VUPf506).

Pistachio seedlings that exposed to nematodes became thicker/weaker over time, with some of them losing their green color and gradually turning pale purple. In contrast, Pistachio seedlings which were not treated with nematode or bacteria grew normally. Pistachio seedlings in the infected control (i.e., the ones inoculated with the 2000 J2 of *M. incognita* but not with bacteria strains) had a lower growth rate in

plant height, root length, fresh weight, and dry weight compared with seedlings grown in the "normal" condition. In contrast, seedlings inoculated with the juveniles of *M. incognita* and different carrier formulations of *P. fluorescens* strains increased the growth rates significantly compared with the control seedlings. Some bacterial treatments showed less effect on the growth of seedlings

compared with normal seedlings, but they still had positive effect on pistachio seedling growth compared with the control one (Fig 1). There were high rate of growth regarding seedling root in root dry and wet weight in *P. fluorescens* strain

treatments in wheat bran-based formulation. On the other hand, the formulations (wheat bran, talc) of *P. fluorescens* isolate 52+506 (2+C), significantly increased seedling growth over the control ones.

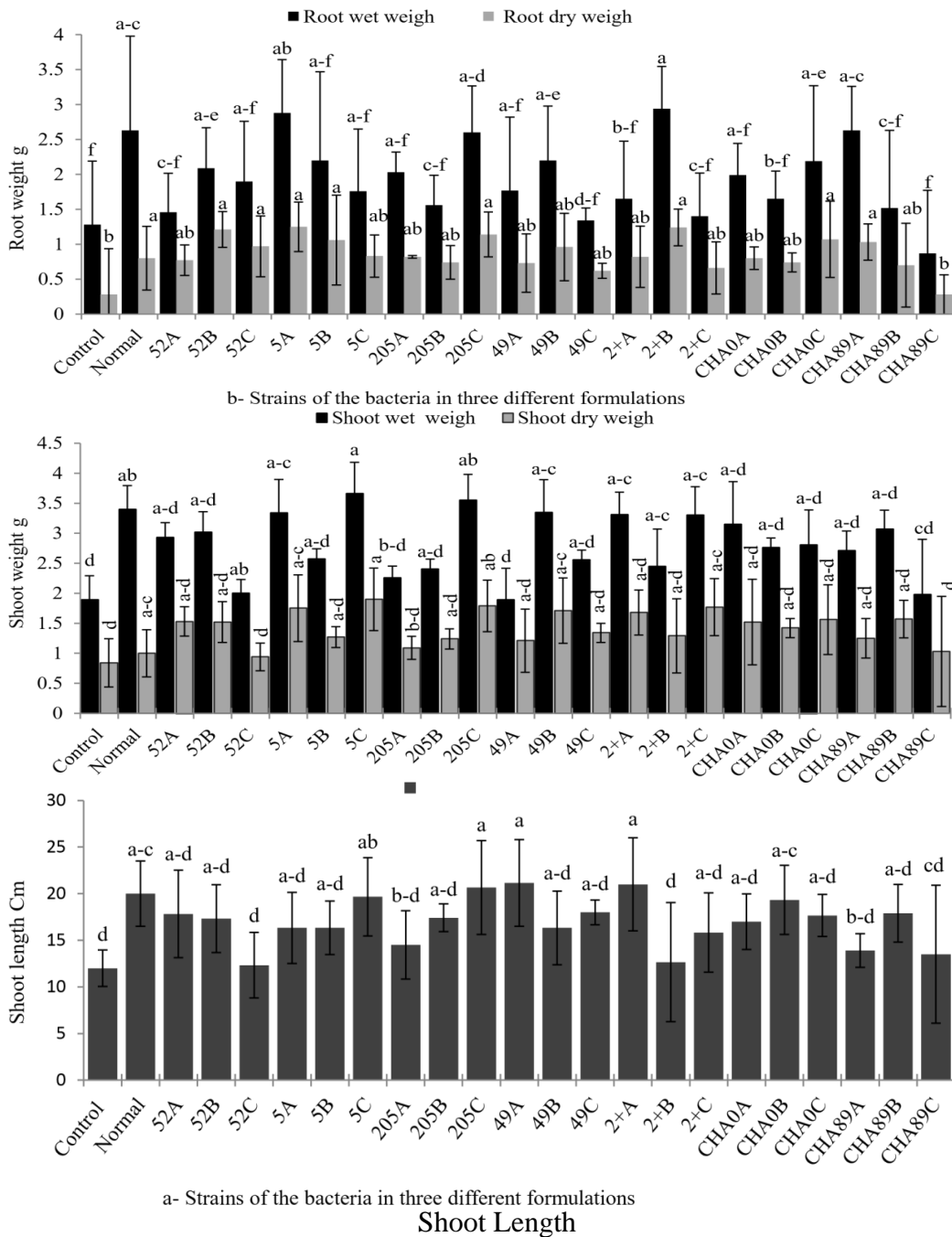


Figure 1. Effect of different types of three carrier formulations of *P. fluorescens* on plant growth traits in pistachio inoculated with the 2000 J2 of *Meloidogyne incognita* in greenhouse experiments (a: Shoot length, b: Root weight and c: Shoot weight)

c- Strains of the bacteria in three different formulations

Standard errors are indicated by error bars. Bars followed by different alphabets are significantly different from each other at $P \leq 0.05$ according to the Fisher's LSD test. Normal represents seedlings inoculated neither with 2000 J2 of *Meloidogyne incognita* nor with *P. fluorescens*. Control represents seedlings inoculated with 2000 J2 of *Meloidogyne incognita* but not with *P. fluorescens*.

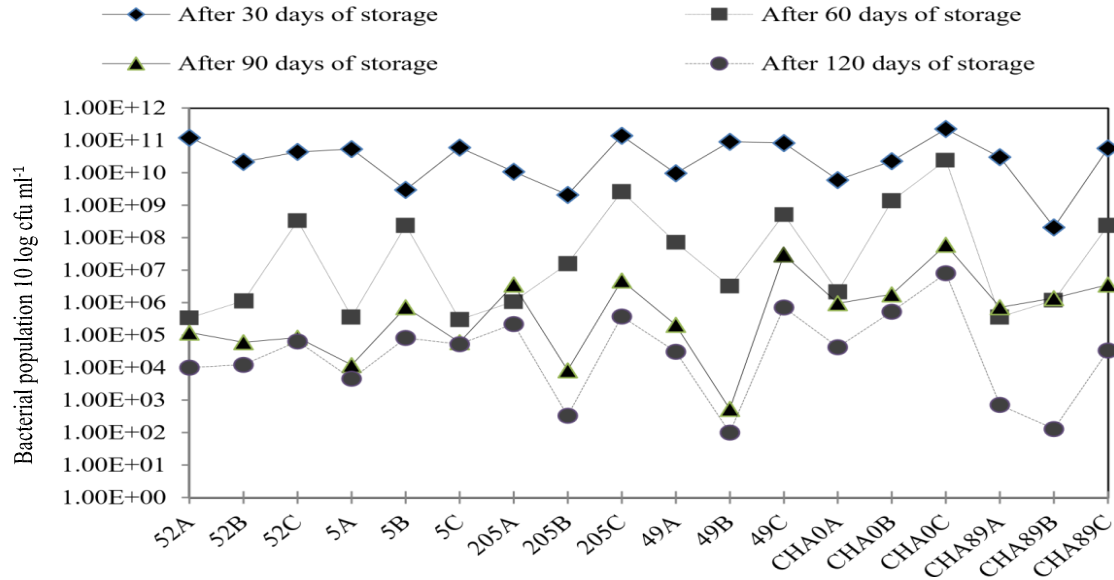


Figure 2. Survival of *Pseudomonas fluorescens* in three carrier formulations within 120 day
Strains of the bacteria in three carrier formulations of fluorescent *Pseudomonads*

Discussion

As far as the authors are concerned, this is the first report of using the powder formulations of native *Pseudomonas fluorescens* to control Pistachio root-knot disease. In this study, *Pseudomonas fluorescens* were able to support and improve the growth of seedlings even in the presence of root-knot nematode. Plant-growth-promotion activities of *P. fluorescens* reported in crops such as, rice, wheat, sorghum pearl millet, tomato, brinjal, chili, red gram, cucumber, and sunflower (Hussain *et al.*, 1990; Umesha *et al.*, 1998; Raju *et al.*, 1999). The population of bacteria shows that they could be established in pistachio seedlings' rhizosphere. When seedlings were inoculated, bacteria established well in the rhizosphere. For the effective control of any soil-borne disease, the introduced antagonist should colonize roots. The successful antagonists should colonize the

rhizosphere at the time of growing of new root growth. In other words, the antagonist should be moved from the beginning of the rhizosphere to its end and be established there (Weller 1984). *P. fluorescens* VUPf52 and VUPf5 were isolated from peach rhizosphere (Khatamidoost *et al.*, 2014).

There are several advantages in the carriers based inoculants of bacteria such as increased shelf life, protection from unsuitable condition etc. (Sangetta *et al.*, 2012). Talc-based formulation of *P. fluorescens* was found to be effective for seed treatment and foliar application in controlling rust and leaf spots of groundnut (Meena *et al.*, 2002), but it did not work for some diseases like damping-off in cotton, and in root-knot nematode on pistachio seedlings like the other new bio-formulations that are tested in this study. It was reported that plant-growth-promoting rhizobacteria (PGPR) survived in certain dry

formulations (Suslow 1982). Populations of PGPR did not decline in the talc mixture after 30 days' storage at 4°C. (Kloepper and Schroth 1981), also, similar result was found after 2 months' storage.

In this study, the population of bacteria did not decline from inoculation to assessment, which indicates the ability of carriers to protect bacteria's longevity. Root zone application of wheat bran-based and the others of *P. fluorescens* formulation increased rhizosphere's population of the bacteria. Certain strains of *Pseudomonas fluorescens* have been shown to provide biological control of root pathogens when they have been applied to soil (Burr and Caesar, 1984), since some of these strains have the ability to colonize the roots (Parke 1990). The yield of pistachio seedlings improved by treatment with *P. fluorescens* formulations. This may be because the formulation suppresses yield loss of various pathogens like root-knot nematode. Root-zone application effectively controlled pistachio root knot and various diseases (O'Sullivan and O'Gara 1992).

Among the various tested formulations, wheat bran-based is the best formulation of *P. fluorescens* strains which may contain a suitable nutrient medium for bacteria growth, was used for the first time in this study.

The real characteristics of carrier include more surface area, potential of organic matter, high ability in holding water, easy availability, price and normal pH (Arangarasan *et al.*, 1998). Wheat-bran carrier is general, cheap and found everywhere. Moreover, the combination of these two strains of *P. fluorescens* (VUPf52+VUPf506) had a great effect on disease reduction on root-knot nematode and were effective in increasing seedling growth. These results were also, found by others (Khodakaramian *et al.*, 2008). The results of the present study may have practical applications in disease control; it is necessary to test these results on the field. As already noted, formulation and establishment of biocontrol, agents are very important for their effectiveness. Wheat bran-based formulation developed and tested in the greenhouse condition can test for controlling pistachio root-knot nematode in field. In greenhouse condition, it has the potential to replace chemical nematicides (unpublished data) and to be utilized as an important component of IPM (integrated pest management), which is a promising approach to a sustainable agriculture.

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