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.,
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 steam-sterilized at 140 KPa for 30 min, and dried aseptically in glass trays for 12 h at 50°C. Each carrier material was kept in storage condition of 28 to 33 °C on a laboratory bench for testing greenhouse studies and survival activities.

<table>
<thead>
<tr>
<th>carrier/Treatments</th>
<th>Ingredients/Methods</th>
<th>Strains/s</th>
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<tbody>
<tr>
<td>Talcum powder (A)</td>
<td>Suspension of <em>P. fluorescens</em> strain (400ml) containing $1\times10^{12}$ CFU ml$^{-1}$ mixed with talcum powder (100g) and CMC (25g).</td>
<td>VUPf52, VUPf5, VUPf506, VUPf5205, VUPf49, CHA0, CHA89</td>
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<tr>
<td>Dried Apple Powder (B)</td>
<td>Suspension of <em>P. fluorescens</em> strain (400ml) containing $1\times10^{12}$ CFU ml$^{-1}$ mixed with dried apple powder (50g), talcum powder (50g) and CMC (10g).</td>
<td>VUPf52, VUPf5, VUPf506, VUPf5205, VUPf49, CHA0, CHA89</td>
</tr>
<tr>
<td>Wheat Bran (C)</td>
<td>Suspension of <em>P. fluorescens</em> strain (400ml) containing $1\times10^{12}$ CFU ml$^{-1}$ mixed with wheat bran (50g), talcum powder (50g) and CMC (25g).</td>
<td>VUPf52, VUPf5, VUPf506, VUPf5205, VUPf49, CHA0, CHA89</td>
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</table>

2+A Suspension of *P. fluorescens* strains (400ml) containing $1\times10^{6}$ CFU ml$^{-1}$ of each strain mixed with talcum powder (100g) and CMC (25g).
| 2+B | Suspension of *P. fluorescens* strains (400ml) containing $1\times10^{6}$ CFU ml$^{-1}$ of each strain mixed with dried apple powder (50g), talcum powder (50g) and CMC (10g). | VUPf506+VUPf52 |
| 2+C | Suspension of *P. fluorescens* strains (400ml) containing $1\times10^{6}$ 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 $1 \times 10^{12}$ 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 $1 \times 10^{12}$ 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 ± 2°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 ± 2°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 $1 \times 10^{12}$. Although, subsequently, there was a decline in population, 120 days after incubation the bacterial population was $1.3 \times 10^5$ to $8.9 \times 10^6$ per gram in dried apple-based and wheat bran-based formulations (Fig 2).

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

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

<table>
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<tr>
<th>Treatments</th>
<th>Gall</th>
<th>G.L.</th>
<th>Egg Mass</th>
<th>R.F.</th>
<th>CFU ml$^{-1}$</th>
<th>Disease Reduction (%)</th>
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<tr>
<td>Control</td>
<td>54.0</td>
<td>4.3</td>
<td>59.0</td>
<td>0.98</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>52A&lt;sup&gt;e&lt;/sup&gt;</td>
<td>18.0</td>
<td>3.4</td>
<td>22.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.14&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.16×10&lt;sup&gt;7&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
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</tr>
<tr>
<td>52B</td>
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<td>2.3</td>
<td>6.8&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.14&lt;sup&gt;d&lt;/sup&gt;</td>
<td>8.83×10&lt;sup&gt;8&lt;/sup&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
<td>84.9</td>
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<td>5.0&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.33&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
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<td>3.2</td>
<td>22.9&lt;sup&gt;d&lt;/sup&gt;</td>
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<td>0.05&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6.94×10&lt;sup&gt;6&lt;/sup&gt;&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>32.7</td>
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Each value is mean of four replicates; values within a column followed by different alphabets are significantly different from each other at *P*≤0.05 according to the Fisher’s LSD test. <sup>a</sup>Gall index. <sup>b</sup>Reproduction factor = Pf/Pi (Final population/Initial population). <sup>c</sup>Control represents seedlings inoculated with *Meloidogyne incognita* 2000 J2 but not with *P. fluorescens*. <sup>d</sup>Three carrier formulations of *P. fluorescens* (A: talcum powder, B: dried apple powder and C: wheat bran). <sup>e</sup>Mixture of two strains (VUPf52 and VUPf506).
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)
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 fined 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. The authors acknowledge University of Guilan for providing research funds, Vali-e-Asr University of Rafsanjan to provide bacterial isolates and Iran’s Pistachio Research Institute (IPRI) for providing the research material.

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