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Potency evaluation of *Pseudomonas aeroginosae* and *Pseudomonas fluorescens* as biocontrol agents for root-knot nematodes in Iran

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Abstract: Biocontrol agent, Nematodes, *Pseudomonas* sp.

Biological control is considered as new efficient method that becomes widely used for controlling plant parasitic nematodes, as aim to decrease the extent of environment degradation and the effect of the excessive toxic nematicides. So, this study was done to investigate the role of some bacterial genera as biocontrol agent against *Meloidogyne incognita*. A number of bacterial species, isolated from root-knot nematodes conductive soil of different localities Guilan Province in Iran, were evaluated for suppression of *Meloidogyne incognita*. Nine isolates bacterial isolates significantly reduced nematode larvae population in soil belonging to *Meloidogyne* spp. Death percentage of nematode larvae ranged from 20.7% to 76.25%. Five potent bacterial isolates with higher nematocidal activity were selected and identified as *Pseudomonas aeroginosae* and *Pseudomonas fluorescens* using morphological and biochemical diagnosis tests The study indicated that *Pseudomonas aeroginosae* and *Pseudomonas fluorescens* were potent as bio-control agents for root-knot nematodes, the production of local Iran inoculums of both bacterial species as a safe bio-control agents for the root-knot disease is possible.

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Introduction

The continuing world population pressure ensures the need to maximize the average yields of most major crops (Ashoub and Amara, 2010). Crops production in greenhouses is a method for economically maintaining a warm environment during cool seasons and to protect crops, especially vegetables, from unfavorable weather conditions. However, root-knot nematodes and soil borne fungi greatly affect plant growth and yield in greenhouses (LI Bin *et al.*, 2005). Root-knot nematodes are widespread and important pests of both annual and perennial crops in Iran and other countries, where they cause extensive damage to fruit and vegetable crops (Mohamed, 2009). The infection starts with root penetration of second stage juveniles hatched in soil from eggs encapsulated in egg masses laid by the females on the infected roots. The interaction between the root infecting fungus and the nematode results in the reduction of seed emergence and increase in both galling and nematode fecundity, with the results that the population of *M. javanica* increased in the presence of *Rhizoctonia solani*. Simultaneously, the disease development caused by soil-borne fungal pathogens was also stimulated by nematode on soy bean (Dawar *et al.*, 2008).

Management of the disease has relied largely upon pesticides application. The frequented and extensive use of chemical insecticides has resulted in developing a wide spread resistance of certain pathogens to a range of pesticides (Butter *et al.*, 2003). The divesting implication of pesticides resistance developing in insect populations combined with the increased global interest to reduce the input of harmful pesticides has encouraged research into the development of environmentally benign strategies for pest control including the use of microbial species (Atkins *et al.*, 2004). Bacteria, yeast and filamentous fungi are common inhabitants of soil and plant surfaces, by various mechanisms, they may affect the growth of pathogens and reduce the disease they cause. The predominant (Leite *et al.*, 2005) nematodes in soils and roots of crops were of the genus *Meloidogyne* (Ibewiro *et al.*, 2000). The biological control of root-knot nematode *Meloidogyne java* was investigated using several strains including *Trichoderma harzianum, Metarizium anisopliae* and *Baeuvaria* sp. (Small *et al.*, 2005). Limited numbers of bacterial species have been reported as biological control agents for root-knot nematode disease. Some bacterial species with nemacidal activity has been used with some success for controlling against root-knot diseases including *Streptomyces* spp., *Serratia* spp., *Bacillus* spp., *Azotobacter chroococcum, Rhizobium, Corynebacterium* and *Pseudomonas* (Hallmann *et al.*, 2001).

Hanna *et al.* (1999) evaluated *Pseudomonas fluorescens* for the control of *Meloidogyne incognita* on tomato plants. They found that the percentage of gall formation and root gall index were decreased when the bacteria were introduced prior to nematodes. Siddiqui and Shaukat, (2002) noted that *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* reduced *M. javanica* juvenile penetration into tomato plants. In 2004 El- Hamshary *et al.* found that *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* affected *M. incognita* juveniles' survival *in vitro* study, and the mortality percentages of the nematode were dependent on the bacterial concentration and exposure time. Moreover, Siddiqui and Shaukat in 2004 concluded that fluorescent pseudomonads induce systemic resistance against *Meloidogyne javanica* via signal transduction pathways which is independent of salicylic acid accumulation in roots. Concerning *Rhizobium*, Sidiqqui *et al.* in 2001 noted that, this genus can significantly reduce egg hatching and caused mortality of *M. javanica in vitro*.

In a screening programed, two bacterial isolates of 33, isolated from the rhizosphere of wild and cultivated plants caused significant (>50%) mortality of *Meloidogyne javanica*, the root-knot nematode juveniles *in vitro*. In the same study, selected strains of the antagonistic rhizobacteria reduced nematode penetration and subsequent root-knot infection in mungbean (Hamid *et al.* 2003). The objectives of the

present study were: isolation of local bacterial strains that suppress root-knot nematodes, identification of the most potent isolates and evaluation of the potent antagonistic isolates for controlling of *Meloidogyne* under *in vitro* conditions.

Materials and methods

Sampling and nematode extraction

Samples were collected in infested orchards. Each sample consisted of dozens of tiny sub samples collected at 15 - 25 cm depth and 20 cm distance from the crown. The samples, one and a half pounds of plant and ten gram plant roots, were later transferred to the laboratory.

The nematode separation method was used (Jenkins 1964), and centrifugal separation was performed according to the method of Coolen and D' herde (1972), from collected roots. Isolation of antagonistic bacterial strains: A total of 40 bacterial strains were isolated from the rhizosphere of tea plants from the Guilan province (North of Iran). All isolates were cultured on both nutrient agar and King's B media. In brief, one gram of soil was suspended in 100 ml sterilized distilled H_2O containing one gram of gelatin and then shacked for 30 minutes at 70 rpm. The resultant suspensions were diluted up to $1x10^7$and streaked on agar media and kept at 27±1°C for 72 h. Bacterial colonies were purified and stored at 4 °C for further investigation.

In vitro evaluation of antagonistic activities of the bacterial strains against root-note nematodes

Bacterial suspensions were prepared in sterilized distilled water adding 1 ml from each suspension to 100 ml nutrient broth or King's B broth, later allowed to grow under shaking for 48h at 25°C. The cultures were centrifuged at 5000 rpm for 15 min and the supernatants were evaluated for anti-nematicidal activities of tested bacteria against *M. javanica*. To perform the test, a total of 30 *M. javanica* active juveniles were added into 1 ml of each bacterial supernatant and incubated at 27-29°C for 48 h. Sterilized distilled water was used as control. The experiment was conducted in a randomized completely design in three replicates and following formula was used to calculate percentage of nematode juvenile mortality, as normalized on controls.

$$\text{Mortality (\%)} = \frac{[C1-C2]}{C1} \times 100$$

Where, C_1 is the number of live nematodes juveniles in control treatments and C_2 is the number of live nematodes juvenile counted in other treatments.

Phenotypic characteristics of the bacterial strains

The most effective bacterial strains were selected and their phenotypic features were characterized based on the standard bacteriological methods (Schaad *et al.,* 2001).

Results

Isolation of antagonistic bacterial strains

Antagonistic activities of the challenged bacterial strains were determined based on juvenile mortality. The strains nematicidal activities were quite variable ranking from 20.7 to 75.24%. Among the 5 tested *Pseudomonas* strains, 4 strains of *P. fluorescenes* (RH-79 and RH-37) showed high levels of antagonistic activity (Group A). Within this group, *P. fluorescenes* biovar VI (RH-79) ranked first causing 75.24% of juvenile mortality (Table 1). Strains RH-25 and RH-37 showed 63.58% and 70.44% nematicidal activities, respectively.

Phenotypic features determination of the bacterial strains

Based on rates of nematicidal activities of the bacterial strains, 5 isolates were chosen for further characterization, based on Schaad *et al.*, 2001 (Table 2).

Discussion

Biological control of soil-borne pathogens by rhizosphere bacteria is notoriously susceptible to alterations in experimental conditions (Deacon, 1991; Weller, 1988). Among rhizosphere nematode antagonists, the Gram+ *Pasteuria penetrans* is an antagonist specialized against root knot nematodes (Daudi *et al.* 1990; Kloepper and Tuzun, 1995; Shanthi *et al.,* 1998). Beside this bacterium, also

nematode trapping fungi can reduce populations of nematodes (Mohotti *et al.,* 1998).

In this study, five isolates belonging to the genus *Pseudomonas* were found to possess a pronounced nematicidal activity. Almost all selected isolates showed similarities in diagnostic properties with *P. fluorescens*, whereas only Rh-25 was identified as *P. aeroginosa*. *Pseudomonas fluorescens* and *P. aeruginosa* showed variable antagonistic activities against *M. incognita*, reducing its juvenile in range by 40.26 – 75.24 %.

Table 1. *In vitro* antagonistic activities of 34 rhizosphere bacteria of tea plants against *Pratylenchus loosi* based on juvenile mortality.

Strain	Mortality (%)	Statistical group
Rh-79	75.24	A
Rh-37	70.44	A
Rh-39	52.98	B
Rh-19	40.26	C
Rh-44	34.24	D
Rh-94	24.25	DE
Rh-31	20.01	E
Control	15.63	F

Data with different letters show significant differences based on Duncan's multiple range test (α= 0.05).

These findings are new for Province Guilan in Iran. In previous studies (Shanthi *et al.*, 2003) soil application of *P. fluorescence* similarly reduced soil and root population's nematodes. Fluorescent products by *Pseudomonas* were found to have inhibitory effect on hatching and penetration of nematodes and on pigeon pea roots colonization (Siddiqui *et al.,* 2005).

Based on statistical differences observed the isolates of *P. fluorescence* showed different effects, as these bacteria affected nematodes conferring them a different appearance and colors, ranging from brown, to black some specimens appearing also degenerated.

Table 2. Characteristics of eight antagonistic *Pseudomonas* strains against *M. incognita*.

Properties	RH-25	RH-37	RH-39	RH-79	RH-19
Fluorescent pigment	-	+	+	+	+
Oxidase	-	-	-	-	-
Pectolytic activity	+	-	-	-	-
Nitrate to nitrite	+	+	-	-	-
Gelatin liquefaction	+	+	-	+	+
Growth at 41°C	+	+	+	-	-
Growth at 4°C	-	-	-	+	+
Growth at pH 5.7	-	+	-	-	-
Growth in 7% NaCl	-	-	-	+	+
Growth on: Glucose	+	+	+	+	+
D-galactose	-	+	-	+	+
Saccharate	-	-	-	+	+
Xylose	-	+	+	+	+
Arabinose	-	+	+	+	+
Sorbitol	-	+	-	+	+
Mannitol	+	+	+	+	+
Arginine	-	-	+	-	-
L-tryptophan	+	+	+	+	-

+: Positive Reaction; - : Negative Reaction.

According to Westcott and Kluepfel (1993), prior applications of *P. fluorescens* prevented egg hatching and affected juveniles due to exotoxin formation and disruption of normal cellular nematode metabolism. It is important to note that some of these bacteria induce plant systemic resistance for indirect control of soil pathogens, in addition to exhibited antibiosis (Ashoub and Amara, 2010).

Some bacterial species with nematicidal actuality have been applied for control of root-knot nematodes: among them *Streptomyces* spp., *Serratia* spp., *Bacillus* spp., *Azotobacter chroococcum*, *Rhizobioum*, *Corynebacterium* and *Pseudomonas*. Eapen reported that treating pepper seedlings with isolates of *P. fluorescens* reduced the detriment effects due to *Meloidogyne incognita*. Similarly, insemination of wheat plants with *P. fluorescens* terminated in considerable lower nematode populations (Zeinat *et al.,* 2009).

Table 3. The degree of nematicidal activities of effective antagonistic bacteria based on % of juvenile mortality.

Bacterial strain name	Mortality (%)	Significance
P. fluorescent bv. I (Rh-79)	75.24	A
P. fluorescent bv. IV (Rh-37)	70.44	A
P. aeroginosa (Rh-25)	63.58	AB
P. fluorescent bv. V (Rh-39)	52.98	B
Control (distilled water)	15.63	C

It is significant to point that rhizosphere of antagonistic plants may represent beneficial sources of potential biological control agents for nematodes (Kloepper *et al.*, 1992) as suggested by prevention effects of *P. fluorescens* on *M. incognita*. However, this biovar proceeded from radish rhizosphere host for *Meloidigyne* spp. (Ashoub and Amara, 2010).

The results herein showed may represent a fraction of the effects related to the complex relationships among different types of microorganisms in the rhizosphere. PGPR species alone or with *Rhizobium* enhanced plant growth both in *M. javanica* and inoculated plants. Inoculation with *Rhizobium* spp. caused an increase in plant growth than the effect caused by any species of PGPR in nematode-inoculated plants. Combined use of *Rhizobium* with other species of PGPR also decreased galling and nematode propagation than their single inoculation (Siddiqui *et al.*, 2007).

These preliminary results provide a strong incentive for further experiments on the use of rhizosphere bacteria in the biocontrol of plant parasitic nematodes. If the potential of these strains is confirmed, they could be used in the future in greenhouse and field conditions, to develop alternative, low cost and environment friendly technologies.

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