



## RESEARCH PAPER

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## Evaluation of antagonistic *Pseudomonas* against root lesion nematode of tea

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### Abstract

The tea root lesion nematode, *Pratylenchus loosi*, has been shown internationally to be a serious nematode pest causing yield losses in tea plantations. It is one of the most important crop loss agents in Iran, which affect quantity and quality of tea leaves. Biological control agents represent an innovative alternative to agrochemicals and have potentials for control of plant parasitic nematodes. To evaluate these potentiality more than forty bacterial strains were collected from rhizosphere of tea plants and screened for their antagonistic activities towards adult and juvenile *Pratylenchus loosi* for population density reduction under *in vitro* condition. Eight selected isolates with nematicidal activity were characterized and identified. All belonged to the genus *Pseudomonas*. Seven strains were identified as *Pseudomonas fluorescens* and one as *P. aeruginosa*. Death percentage of juveniles ranged from 63.10% to 95.24% for *P. fluorescens* (Rh-36) and *P. fluorescens* (Rh-19), respectively.

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## Introduction

Tea, *Camellia sinensis* (L.) O.Kuntze, cultivated on 2.85 million ha, with a total production of 3.87 million ton per annum. Tea is considered as a strategic economic crop in Iran. According to FAO statistics in 2010, tea is already harvested in Iran from a surface of about 32000 ha (FAO, 2011). This plant is attacked by more than 30 animal species. Amongst the various constraints to tea production, plant parasitic nematodes have a significant economic importance (Lus et al., 2005). As a permanent crop grown as a monoculture, tea creates a stable micro-climate and provides a uniform food environment for several pests and diseases. More than 40 species of plant parasitic nematodes, belonging to 20 genera, have been reported from tea worldwide (Campos et al., 1990). Two species of root-lesion nematodes (RLNs), *Pratylenchus loosi* Loof 1960 and *P. brachyurus* (Godfrey) Godey, are known to attack tea plants in some producing countries such as Sri Lanka, Philippines, Japan, China, Bangladesh, Taiwan, India, Vietnam, USA and Australia (Gnanapragasm et al., 1993). Among these species, *P. loosi*, was seen for the first time in 1930 by Gadd in tea gardens in Sri Lanka and in 1960 was reported by Loof (Sivanpalan, 1972). This nematode caused a severe damage on tea plants and remarkably reduced crop yields in many other countries such as India, China, Japan and Bangladesh (Campos et al., 1990). *Pratylenchus loosi* is a serious parasite of tea in Iran (Pourjam et al., 1999, Seraji, 2007), causing losses in tea quantity and quality (Seraji et al., 2010).

The side undesired effects of common pesticides lead the investigators to develop and apply environmentally safe pest management strategies, including microbial-based compounds. Bacteria, yeast and filamentous fungi are general inmates of soil and plant surfaces, and some species are known for various mechanisms limiting disease incidence or severity (Zhang et al., 2000, Vey et al., 2001, Leite et al., 2005, Quesada and Vey, 2004, Small and Bidochka, 2005, Vizcaine et al., 2005, Steddom and Menge, 2001, Atkins et al., 2003, 2004).

Various management systems have been designed to envisage and introduce more efficient compounds against plant-parasitic nematodes, notably in the past thirty years (Duncan, 1991, Barker and Koenning, 1998). In general, invoking chemical nematode management when it is unavoidable, or strictly needed, aims at reducing the responsibility and effects of the synthetic agrichemicals on the environment (Kimpinski et al., 2001). The rhizoplane and rhizosphere are colonized and differently affected by many microorganisms. Plant growth promoting bacteria supply plant growth promoting matter and antibiotics. They prepare fundamental guarding against nematode diseases (Abuzar and Haseeb, 2010). Up to 10% of rhizobacterial populations have been shown to be antagonist on parasitic on nematodes. However the application of crop rotations and mulches as a procedure to increase levels of rhizoflora antagonists to plant-parasitic nematodes showed variable results (Oostendorp and Sikora, 1986, Kloepper et al., 1992, Westcott and Kluepfel, 1993, Pedersen et al., 1988).

The nematicidal activities of these bacteria may be attributed to antibiotics produced in the agar medium. The seed or tuber treatments with non-parasitic rhizobacteria and even their application in soils may affect root penetration by nematodes on diverse crops, both in greenhouse and field conditions. Use of these non-parasitic rhizobacteria among other beneficial microorganisms such as root-nodule bacteria, arbuscular mycorrhizae, saprophytic and opportunistic fungi appeared advantageous for suppression of nematode populations on various crops (Becker et al., 1988, Oostendorp and Sikora, 1990, Siddiqui and Mahmoud, 1993, 1995a, 1995b). *B. megaterium* was reported to reduce by 50% penetration of both *M. chitwoodi* and *Pratylenchus penetrans* in potato (Al-Rehiyani et al., 1999).

To best of the author's knowledge no single data is available on biocontrol by *Pseudomonas* of *Pratylenchus loosi* on tea plants. Recently we have demonstrated the application of four *Bacillus subtilis*

strains which suppressed the tea nematode and lead to juveniles death ranged from 62.88% to 86.01%(Rhanandeh et al.,2012).

In current study we further isolate and characterize some native bacterial strains capable to suppress tea root-lesion nematodes, under laboratory condition.

## 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 tea and ten gram tea roots, were later transferred to the laboratory.

The tea root lesion 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 strain*

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<sub>2</sub>O containing one gram of gelatin and then shaken for 30 minutes at 70 rpm. The resultant suspensions were diluted up to 1x10<sup>7</sup> 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-lesion 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 *P. loosi*. To perform the test, a total of 30 *P. loosi* 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<sub>1</sub> is the number of live nematodes juveniles in control treatments and C<sub>2</sub> 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).

### *Protease test*

This test was carried out using skim milk agar (casein peptone 5g, yeast extract 5 g, skim milk 1 g, glucose 1g and agar 10.5 g per liter). Bacterial strains were inoculated on casein agar medium and the plates were incubated at 27 °C for 48 hours. The clear zones around the colonies were considered as positive reaction (Olajuyigbe and Ajele, 2005).

## 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 14.15 to 95.24%. Among the 34 tested *Pseudomonas* strains, 4 strains of *P. fluorescences* (RH-36, RH-25, RH-79 and RH-37) showed high levels of antagonistic activity (Group A). Within this group, *P. fluorescences* biovar I (RH-36) ranked first causing 95.24% of juvenile mortality (Table 1). Strains RH-79, RH-25 and RH-37 showed 84.98, 91.90 and 87.44% nematicidal activities, respectively.

*Phenotypic features determination of the bacterial strains*

Based on rates of nematicidal activities of the bacterial strains, 8 isolates were chosen for further

characterization, based on Schaad et al., 2001 (Table 2).

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

Strain	Mortality (%)	Statistical group	Strain	Mortality (%)	Statistical group
Rh-36	95.24	A	Rh-77	26.37	FG
Rh-25	84.98	A	Rh-33	20.00	FG
Rh-79	91.90	A	Rh-15	20.00	FG
Rh-96	70.15	BC	Rh-74	22.94	FG
Rh-35	71.17	BC	Rh-12	28.87	EFG
Rh-37	87.44	A	Rh-11	22.28	FG
Rh-19	63.10	C	Rh-76	22.17	FG
Rh-39	82.62	AB	Rh-53	17.15	G
Rh-50	29.15	EFG	Rh-43	27.85	FG
Rh-17	24.39	FG	Rh-85	42.95	DE
Rh-60	26.04	FG	Rh-99	23.68	FG
Rh-57	20.49	FG	Rh-28	33.83	DEF
Rh-31	20.01	FG	Rh-23	34.68	DEF
Rh-63	18.69	FG	Rh-48	44.45	D
Rh-41	22.86	FG	Rh-94	24.25	FG
Rh-78	21.43	FG	Rh-44	34.24	DEF
Rh-16	25.47	FG	Control	15.63	G

Data with different letters show significant differences based on Duncan's multiple range test ( $\alpha = 0.05$ )

#### *Protease test*

Cassese is an exoenzyme which produces by some bacteria to degrade casein. All tested bacterial strains showing antagonistic activity against *Pratylenchus loosi* were able to produce proteases. Among the tested strains three species of *P. fluorescent* bv. IV and *P. aeruginosa* showed the largest clear zones, indicating high level of protease production.

#### **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). According to Maafi (2000) isolates of *Pasteuria penetrans* do not attach to second stage juveniles of *P. loosi*.

A protozoan endoparasite was occasionally registered from *P. loosi*, and its control impact was not confirmed (Luc *et al.*, 2005). For several years, compost and soil modifications have been practiced in a unified management program to suppress *P. loosi* in Sri Lanka. In addition to many other useful effects, these practices were known to enhance

population densities of natural predators and parasites of parasitic nematodes (Visser, 1959,

Sivapalan, 1972, Gnanapragasam, 1991).

**Table 2.** Characteristics of eight antagonistic *Pseudomonas* strains against *Pratylenchus loosi*.

Properties	RH-25	RH-35	RH-36	RH-37	RH-39	RH-79	RH-19	RH-96
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

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

Bacterial strain name	Mortality (%)	Statistical group
<i>Pseudomonas fluorescent</i> bv. I (Rh-36)	95.24	A
<i>P. aeruginosa</i> (Rh-25)	84.98	A
<i>P. fluorescent</i> bv. I (Rh-79)	91.90	A
<i>P. fluorescent</i> bv. IV (Rh-96)	70.15	BC
<i>P. fluorescent</i> bv. IV (Rh-35)	71.17	BC
<i>P. fluorescent</i> bv. IV (Rh-37)	87.44	A
<i>P. fluorescent</i> bv. I (Rh-19)	63.10	C
<i>P. fluorescent</i> bv. V (Rh-39)	82.62	AB
Control (distilled water)	15.63	D

Data with different letters show significant differences based on Duncan's multiple range test ( $\alpha = 0.05$ )

In this study, eight isolates belonging to the genus *Pseudomonas* were found to possess a pronounced nematocidal activity. Almost all selected isolates showed similarities in diagnostic properties with *P. fluorescens*, whereas only Rh-25- was identified as *P. aeruginosa*. *Pseudomonas fluorescens* and *P. aeruginosa* showed variable antagonistic activities against *P. loosi*, reducing its juvenile in range by 63.1 - 95.2 %.

These findings are new for Iran. In previous studies (Shanthi et al., 2003) soil application of *P. fluorescence* similarly reduced soil and root populations of lesion nematodes viz., *Radopholus similis*, *P. coffee* and *Helicotylenchus multicinctus* in comparison with carbofuron 3G. 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.

According to Westcott and Kluepfel (1993), prior applications of *P. fluorescens* prevented egg hatchin 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 nematocidal 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).

It is significant to point that rhizosphere of antagonistic plants may represent beneficial sources of potential biological control agents for nematodes. Kloepper et al., 1992as 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 and 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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