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Inhibition of Tea Root Lesion Nematode, *Pratylenchus Loosi*, by rhizospher bacteria

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Abstract

Root-lesion disease, which is caused by *Pratylenchus loosi*, is one of the most important diseases currently impacting Iran tea plantations. This disease causes great economic crop impacts. Northern provinces, which supply much of Iran's tea production, have been especially hard impact by root-lesion disease.The purpose of this study was to biological control the nematodes as one of the main sections and sustainable agriculture in integrated management systems, allowing application of *Bacillus subtilis* in the rhizosphere of tea plants infected with the root lesion nematode. In order to study this disease, more than fifty bacterial strains were collected from the rhizosphere area of the tea plants and screened for their antagonistic activities on the reduction of the density population of the adult and juvenile of *Pratylenchus loosi* under in-vitro condition. Four selected isolates with nematicidal activities were characterized and identified. All belonged to the species Bacillus subtilis. Death percentage of juveniles ranged from 62.88% to 86.01% for *Bacillus subtilis* (Rh-14) and (Rh-18), respectively. All bacterial strains isolated in this study had the ability to produce protease. The information obtained was needed as the first step toward the search for control strategies of root-lesion disease in tea plantations in Iran.

Keywords: Bacteria, Biological control, Nematode, Tea.

INTRODUCTION

Tea, *Camellia sinensis*(L) D.kuntze, is an edible and evergreen plant which has numerous medicinal and calmative characteristic. This crop is cultivated on 2.85 million ha and with a total production of 3.87 MT (million tone) per annum (FAO, 2010). Among the various constrains to production of tea, plant parasitic nematodes have a significant economic importance (Bridge *et al.*, 2005). The root- lesion nematodes (RLNs), a revulsive endoparasite with a board host range, can cause economic levels of determinate to many crop species (Thies, 1991). This group of nematodes is migratory endoparasite feeding primarily in the cortical parenchyma and thereby causing severe damage on a wide range of crops (Castillo and Vovlas, 2007). *Pratylenchus loosi* Loof. is known to be a parasite of tea plant in some tea-producing countries such as Sri Lanka, Philippines, Japan, China, Bangladesh, Taiwan, Indian, Vietnam, USA and Australian (Gnanrapragasam *et al*., 1993). This nematode is a serious pathogen of tea in Iran (Pourjam *et al.*, 1997a, 1999b; Seraji, 2007), which cause loss in quantity and quality of tea (Seraji *et al*., 2010). Due to the soil tissue in these areas *Pratylenchus loosi* can complete 3-4 life cycles in tea plantations, resulting in the massive epidemics of the disease. The symptoms of tea root-lesion disease are consist of; stunting, wilting, yellowing and the reduction in the root length. Such symptoms are the result of the root-system being eradicated by the organism which causes lesion of the roots. The consumption of chemicals to control root-knot disease has not provided good results (Maafi, 2000). One of these prosperous methods is the biological control, where bacteria represent an important beneficial group for microbial control and is competent for limiting nematode reproduction. Chahal and Chahal (1993) and Zukerman *et al.*, (1993) reported that Bacillus thuringiensis quenched the populations of *M. javanica* and M. incognita. Naghesh *et al*., (2005) certified the importance of genus Bacillus. Their results indicated that, cell-free culture filtrates of B. cereus reduced egg hatching (90%) and caused 100% mortality of juveniles. Siddiqui and Shaukat (2002) noted that *Pseudomonas fluorescent* and *Pseudomonas aeruginosa* reduced *M. javanica* juvenile permeation into tomato plants. El- Hamshary *et al*. (2004) found that *Pseudomonas fluorescent* and *Pseudomonas aeruginosa* affected M. incognita juveniles survival under an in-vitro condition. Use of bio-control agents such as manure, to enhancement microorganisms is effective in reductioning nematode habitance in soil (McSorley, 2001). Many studies have shown that rhizobacteria, *P. aeruginosa* and *B. subtilis*, not only increase the plant growth but also suppress the root-knot putrefaction and nematode compression in the soil (Siddiqui *et al*., 1999; Siddiqui, 2000; Siddiqui, 2001). *B. megaterium* was reported to reduce by 50% penetration of both *M. chitwoodi* and *Pratylenchus penetrans* in potato (Al-Rehiayani *et al*., 1999). Accordingly, this study aimed at detecting the role of some bacterial genera which used individually and their antagonistic effect when used as mixtures against *Pratylenchus loosi* under in- vitro condition.

MATERIALS AND METHODS

Sampling and nematode extraction

Sampling for *P. loosi* extraction was performed from infested tea plantation in the north Iran, during the years 2010-2011. Total number of 20 complex samples was collected from infested tea gardens, yearly. Each sample consisted of dozens of tiny sub samples collected from the depth of 15 - 25 cm and 20 cm distance from the crown. Each sample was consisted of one and a half pounds of the soils round the tea roots area and ten gram of tea roots, were later transferred to the laboratory. Tea root lesion nematode isolation was performed according to the published method (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

More than 80 rhizospher soil samples from tea bushes were collected from infected and non

infected orchards of Guilan province. Samples were collected from the 15 to 20 cm depth of the rhizosphere area. A total of 40 bacterial strains were isolated using serial dilution method on nutrient agar and King's B media. In brief one gram soil aseptically suspended in 100 ml sterilized distilled water containing one gram gelatin and shacked for 30 minutes with 70 rpm. Soil suspensions were diluted using sterilized distilled water (up to 1x107) and one ml of each sample poured aseptically on plates containing NA or King's B media and they were kept at 27±1ºC for 72h. Desired bacterial colony were selected and stroked again on media and finally kept at 4 ºC for further investigations.

Evaluation of antagonistic activity of the bacterial strains against root-lesion nematodes under *in vitro* condition

Bacterials suspension were prepared in sterilized distilled water and one ml of each suspension was added to 100 ml nutrient broth or King's B broth and they were shacked for 48 h at 25 ºC. Bacterial suspension was centrifuged and the supernatant were used for evaluation of their antagonistic activity toward *Pratylenchus loosi*. A total of 30 *Pratylenchus loosi* active juvenile were added into one ml of each bacterial suspension and they were kept at 21-23º C for 48 h. Sterilized distilled water was used as control and larva mortality percentage were recorded for analysis. This experiment was conducted in a randomized completely design with three replications. The following formula was used to calculate the nematode juvenile mortality percentage: Mortality % $= \frac{[C1-C2]}{C1} \times 100$

Where, C_1= Number of live nematodes juvenile in control treatment and C_2= Number of live nematodes juvenile in other treatments.

Phenotypic features determination of the bacterial strains

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

Protease test

This test was conducted using Skim Milk Agar (casein peptone 5 g, yeast extract 5 g, skim milk 1 g, glucose 1g and agar 10.5 g per liter). Bacterial strains were grown on the culture and they were incubated at 27 ° C for 48 hours. The clear zone around the colony of bacterial strains which indicate protease activity was recorded and analyzed (Olajuyigbe and Ajele, 2005).

RESULTS

Isolation of antagonistic bacterial strains

Analysis of the antagonistic activity of the bacterial strains on *Pratylenchus loosi* showed that there was a significant difference in antagonistic potency between isolates. Mortality percentage of nematode's juvenile confined from 62.88 to 86.01%. *Bacillus subtilis* (Rh-18) showed the greatest antagonistic activity against P. loosi (Table, 1 and Fig.1).

Phenotypic features determination of the bacterial strains

Phenotypic features of the bacterial strains were determined based on the Schaad *et al*., (2001) which were shown in Table 2 for Bacillus strains.

Protease test

Casease is an exoenzyme that is produced by some bacteria in order to degrade casein. Casein is a large protein that is responsible for the white color of milk. All tested bacterial strains which showed antagonistic activity against *Pratylenchus loosi* produced proteases. Among the tested strains, *Bacillus subtilis* RH-18, showed largest clear zone which indicate a high level of protease production (Fig. 2).

DISCUSSION

Recently, it has shown that a number of antagonistic plants contain biocontrol activity against nematodes by choosing antagonistic bacterial in rhizoflora and can repress the plant-parasitic nematodes (Westcott and Kluepfel, 1993), and at the same time elevating the plant growth (Hoffman-Hergarten *et al.*, 1997; Kloepper *et al*., 1992; Sikora, 1992; Oostendorp and Sikora, 1986). Identically, fungal endophytes such as *Acremonium coenophialum* have been found to restrict *Meloidogyne* marylandi and Pratylenchus scribneri attack (West *et al.*, 1988).This is the first report of bacterial antagonism against *Pratylenchus loosi*. In the present study, four isolates of Bacillus bacteria possessed a pronounced nematicidal activity. These active isolates showed variation in their potency to reduce nematodes juvenile belonging to *Pratylenchus loosi* ranged from 62.88 to 86.01%. Four strains including Bacillus NJ46, Bacillus Na22, Bacillus NJ2, and Bacillus NJ57 were among the potential control agents that decrease *P. brachyurus* populations as much as 68.1-73.9% (Harni *et al*., 2007). A number of studies have reported direct antagonism by other Bacillus spp. against plant-parasitic nematode species belonging to the genera *Meloidogyne*, Heterodera and Rotylenchulus (Kloepper *et al.*, 1992; Siddiqui & Mahmood, 1999; Insunza *et al.*, 2002; Meyer, 2003; Ruzo, 2005). *Meloidogyne incognita* galling on tomato, cucumber and clover was suppressed following the application of the bacterial soil macerates or root treatment (Zavaleta-Mejia and Van Gundy, 1982). Similarly, Sikora (1988) reported that treatment of sugarbeet with *Bacillus subtilis* controlled *M. incognita*, *M.arenaria* and *Rotylenchulus reniformis*. There have been also some reports indicating to the generation of metabolites by rhizosphere bacteria causes nematode eggs degrading (Westcott and Kluepfel, 1993), decrease egg hatching (Oostendorp and Sikora, 1990), affects the vitalism of second stage juveniles (Becker *et al.*, 1988) and degrades specific root exudates resulting in reduced attraction and penetration (Oostendorp and Sikora, 1989). In this study, all bacterial strains produced protease. RH-18 strain produced the largest halo and RH-27 strains produced the lowest zone (Fig. 2). Previous studies have suggested that microbial proteases may contribute to infection of hosts by degrading the host's protective barriers (Ahman *et al*., 2002; Huang *et al*., 2004).

The most compelling evidences to support microbial proteases as virulence factors derived from the researches on protease deficient mutants (Ahman *et al*., 2002; Tian *et al*., 2006). For nematophagous fungi, it is believed that extracellular serine proteases are involved in several steps of the infection: releasing nutrients for pathogenic growth, facilitating penetration by degrading proteins of the cuticle, and digesting the host tissue. It has also been shown that bacterial proteases can degrade and digest nematode cuticle or even kill the hosts (Ahman *et al*., 2002; Clarkson and Charnley, 1996; Meyer, 2003; Morton *et al.*, 2004). It is well known that the cuticle of nematodes is rigid and composed of proteins and chitin, especially the outer part that is covered by a layer of proteinaceous membrane, which is an effective barrier protecting nematodes from damage from the environment (Tunlid *et al.*, 1994). All bacterial strains isolated in this study had the ability to produce protease. The enzymes produced by these bacteria were able to reduce the nematode population size in the field.

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Tables

Table 1. Antagonistic activity of the bacterial strains isolated from tea rhizospher against *Pratylenchus loosi* in laboratory.

Bacterial Strain Name	Juvenile mortality (%)	Statistical Group
Bacillus subtilis(Rh-18)	86.01	A
Bacillus subtilis(Rh-40)	72.94	BC
Bacillus subtilis(Rh-14)	62.88	C
Bacillus subtilis(Rh-27)	74.47	AB
Control	15.63	D

Table 2. Phenotypic features of the Bacillus strains that are antagonist against *Pratylenchus loosi.*

Bacterial strain	RH-40	RH-14	RH-27	RH-18
Gram reaction	+	+	+	+
Motility	+	+	+	+
Starch hydrolysis	-	-	-	-
Anaerobic growth in glucose broth	+	+	+	+
Growth 45 C	+	+	+	+
Growth 5 C	-	-	-	-
Growth pH 5.7	+	-	+	+
Growth 7% NaCl	+	-	+	+
Growth 1 % NaCl	+	+	+	+
Acid from: Xylose	+	-	+	+
Arabinose	+	+	+	+
Mannitol	+	+	+	+

+: Positive reaction; - : Negative reaction

Figures

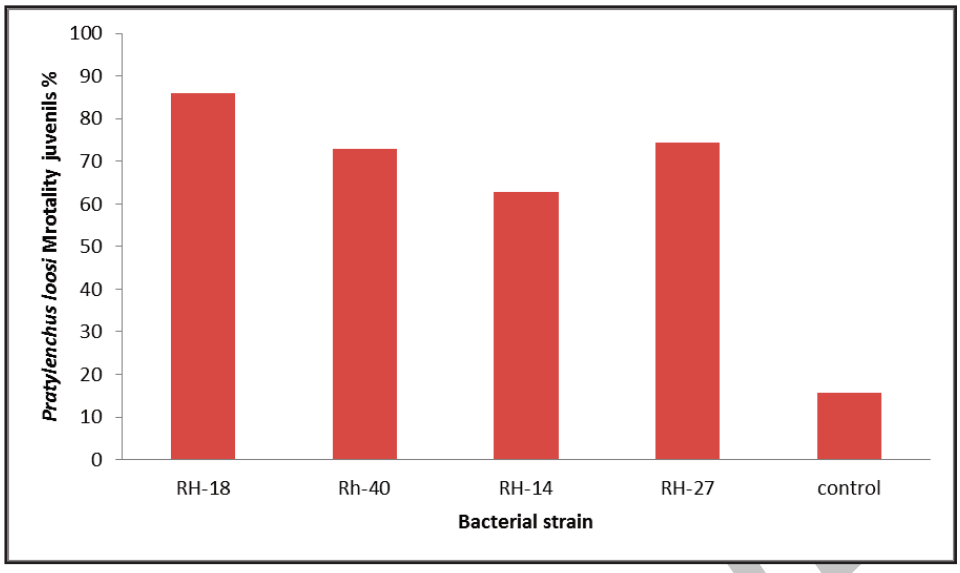

Fig.1. Effect of Cu concentration on the accumulation of Cu in the roots of *A. rosea*

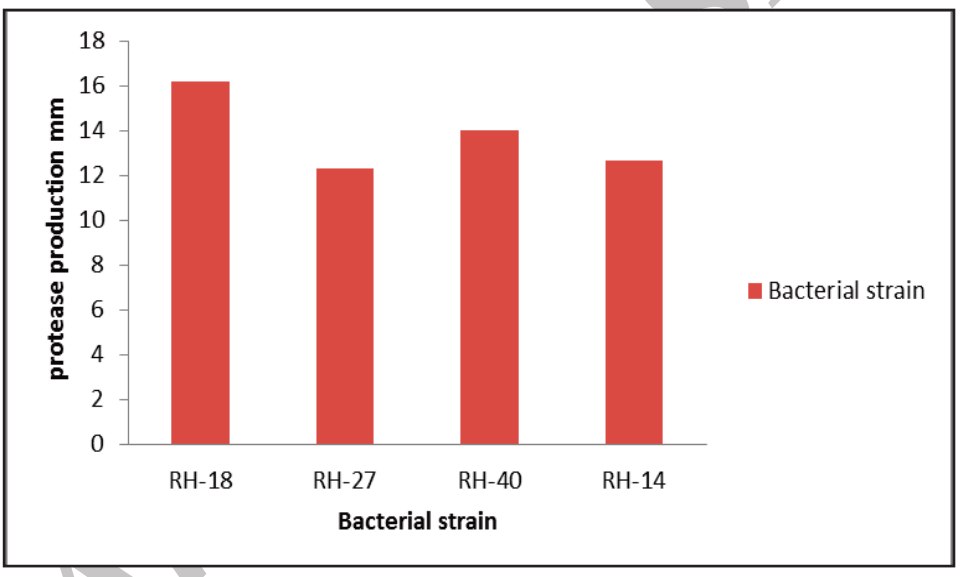

Fig. 2. The aura created by different bacteria.