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# Biological control of the root-knot nematode *Meloidogyne javanica* by *Trichoderma harzianum*

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ABSTRACT

The filamentous fungi *Trichoderma* spp. is currently developed as biocontrol agents against many plant pathogens. Recent studies have shown that these fungi are able to infect nematode eggs and juveniles. In this research, biological control of root-knot nematode (*Meloidogyne javanica*) by *Trichoderma harzianum* BI was investigated in greenhouse and laboratory experiments. Results showed that different concentrations ($10^2$–$10^8$ spores/ml) of *T. harzianum* BI decreased nematode infection and other parameters significantly, compared to control. *T. harzianum* BI was able to penetrate nematode egg mass matrix and significantly decreased nematode egg hatching level. Specific activities of resistance-related enzymes, namely peroxidase (POX), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) increased significantly in *T. harzianum* BI inoculated plants. Maximum activities of POX, PPO and PAL were observed at the 5, 5 and 6 days after inoculation, respectively. Chitinase activity was also increased in culture filtrates of *T. harzianum* BI grown on wheat bran moistened with salt solution supplemented with colloidal chitin or nematode eggs. Maximum activity of chitinase was recorded at the 4 days after inoculation, in media supplemented with colloidal chitin (1.15 U/min per ml) and nematode eggs (0.85 U/min per ml). Results suggested that direct parasitism of eggs through the increase in extracellular chitinase activity, which would be indicator of eggs infection capability, and inducing plant defense mechanisms leading to systemic resistance are two main suppression mechanisms used by *T. harzianum* BI against nematode.

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## 1. Introduction

The root knot nematodes (*Meloidogyne* spp.) are sedentary endoparasites and are among the most damaging agricultural pests attacking a wide range of crops (Mai and Abawi, 1987). Due to the problems caused by chemical control, mainly their deleterious effects on human health and environment, development of alternative control methods is of great importance. *Trichoderma* spp. has been widely studied as a biological control agent against microbial diseases of crops. (Cherif and Benhamou, 1990; Chet, 1987; Chet et al., 1981; Elad et al., 1980, 1983). Several attempts have been made to use *Trichoderma* spp. to control plant parasitic nematodes. *T. harzianum* Rifai has been reported to be an effective bioagent for the management of the citrus nematode (Parvatha Reddy et al., 1996; Rao et al., 1998; Seifullah and Thomas, 1996; Sharon et al., 2001). Windham et al. (1986) reported reduced egg production in the root-knot nematode *Meloidogyne arenaria* following soil treatments with *T. harzianum* and *T. koningii* preparation. Rao et al. (1998) evaluated aqueous extracts of neem (*Azadirachta indica*), caster (*Ricinus communis*) and pingamia (*Pingamia harzianum*) as substrates for the mass production of *T. harzianum* which was used in the management of *Meloidogyne incognita* in eggplant under field conditions. They reported that caster cake extracts showed the best biocontrol activity. Seifullah and Thomas (1996) studied the parasitism of *Globodera rostochiensis* by *T. harzianum* using low temperature scanning electron microscopy. Rao et al. (1998) and Sharon et al. (2001), showed that *T. harzianum* isolates can reduce the *M. javanica* infection.

There is little information on the mechanisms of Trichoderma species against nematodes. Two mechanisms of action are thought to be responsible for the reduction in nematode infection following root treatment with *Trichoderma* spp.: (1) direct parasitism of eggs and larva through the increase in chitinase and protease activities, which would be indicators of eggs infection capability (Sharon et al., 2001; Suarez et al., 2004); and (2) inducing plant defense mechanisms leading to systemic resistance. Extracellular enzymes such as chitinase and protease which display antifungal activities appear to participate in the *Meloidogyne javanica* × *Trichoderma* spp. interaction (Sharon et al., 2001). Chitin is a homopolymer, β-1,4-linked of *N*-acetylglucosamine, the second most abundant

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polymer in nature and also a common structural component (40% w/w) of a nematode eggshell (Wharton, 1980). Nematophagous egg-parasitic fungi, such as *Pochonia chlamydosporia*, must penetrate the nematode eggshell during infection (Lopez-Llorca and Duncan, 1988; Lysek and Krajci, 1987). This structure is formed by several layers (Wharton, 1980), including the chitinous layer, composed of a protein matrix (50–60% of the composition) embedding chitin microfibrils (Kim et al., 1992; Meyer and Wergin, 1998). This layer is probably the major barrier against invasion by soil microorganisms. Furthermore, the collagen-containing cuticle of plant parasitic nematodes also acts as an effective structural barrier to infection (Tikhonov et al., 2002). Parasitic microorganisms must be able to penetrate these barriers for their successful establishment in the host. Suarez et al. (2004) characterized a trypsin-like protease, PRA1, from the culture filtrates of *T. harzianum* CECT2413 grown in liquid medium supplemented with fungal cell wall or chitin. They showed that the number of hatched eggs of root knot nematode *Meloidogyne incognita* was reduced after incubation with pure PRA1 preparation. Khan et al. (2003) showed that the filamentous fungus *Paecilomyces lilacinus* as a biocontrol agent against plant parasitic nematodes penetrates nematode eggs and cuticles through the production of lytic enzymes. A serine protease and chitinase were purified from a culture medium and were further confirmed by *in vitro* tests.

In the present study, we used greenhouse experiments to show the ability of *Trichoderma harzianum* BI to: (1) suppress the root knot nematode, *Meloidogyne javanica* infection and nematode egg hatching on tomato plant; and (2) laboratory experiments to monitor the induction of some resistance-related enzymes in plants and production of extracellular chitinase using colloidal chitin as the substrate.

## 2. Materials and methods

### 2.1. Nematode inoculum preparation

Infected sample was collected from a tomato field in Shiraz (Fars province, Iran) and single egg mass was used to establish a population on Rutgers tomato variety for the experiment. Eggs were extracted from infected tomato roots using 1% NaOCL. Extracted eggs were gently washed with tap water to remove NaOCL (Hussey and Barker, 1973). The species of nematode was identified as *M. javanica* according to morphological and morphometrical characters (Eisenback, 1985).

### 2.2. Fungal inoculum preparation

*Trichoderma harzianum* BI was obtained from plant pathology department, agriculture faculty of Tehran University and was cultured on potato dextrose agar (PDA, Oxoid). Six days after incubation (27°C), the purified fungi were used to produce spore suspension for inoculation.

### 2.3. Plant material

Experiments were carried out with tomato (*Lycopersicon esculentum* var. Roma VF, susceptible to *M. javanica*) grown in a controlled-environment cabinet. The air temperature was maintained at 27°C.

### 2.4. Effect of various concentrations of T. harzianum BI on M. javanica infection

Seeds of tomato were surface sterilized with 1% sodium hypochlorite (5 min) and sown in pots (1 kg) containing sterile soil [Mixture of field soil (silty clay; 78% organic matter) and sand (1:1 v/v)]. Tomato seedlings at four-leaf stage were inoculated with various concentrations of Trichoderma spores (at $10^2$–$10^8$ spores/ml) by root dipping (5 min) respectively. After 2 days, seedlings in each treatment were inoculated with 2000 *M. javanica* Juveniles per individual seedling. Thirty days after seedling inoculation with nematode, number of galls and egg masses per seedling, and number of eggs per individual egg mass were evaluated. Tomato seedlings inoculated with nematode without fungal inoculation [inoculated with sterile distilled water (SD/water)] were used as control. The experiment was performed with 10 replications.

### 2.5. Effect of T. harzianum BI on nematode egg hatching

Tomato seedlings at four-leaf stage were inoculated with 2000 nematode J2 per individual seedling. After 2 days (for penetration of nematode J2 into roots), plant roots were washed with top water and were inoculated with $10^6$ spores/ml of fungus (SD/water as control) by root dipping (5 min) and transplanted in sterile soil. After 20 days, nematode eggs were extracted and were hatched according to Hussey and Barker (1973). Nematode eggs hatching percentage were evaluated for 9 days, and 1 day intervals in treatments and their controls.

### 2.6. Resistance-related enzymes assays

Seeds of tomato were surface sterilized as mentioned above, and sown in pots containing sterile soil. The seedlings were inoculated with $10^6$ spores/ml of fungus at four-leaf stage by root dipping (5 min) and transplanted to sterile soil, and were irrigated with SD/water. Two days after inoculation with fungus, plant roots were inoculated with 2000 nematode $J_2$/plant. Root sampling were done 7 days with 1 day intervals. Fresh tomato roots were washed and dried with filter paper after sampling and homogenized with liquid nitrogen in an ice cold mortar and pestle. The homogenized tissue was rinsed with the same volume of 10 mM sodium phosphate buffer (PH 6.0) at 4 oC, and was filtered through a 0.2 mm nylon filter into a centrifuge tube. The tissue extracts were centrifuged at 12,000 rpm for 20 min at 4 oC. The supernatant was used for the enzymatic activity assay. The experiment was performed with five replications.

### 2.7. Phenylalanine ammonia lyase function assay

Phenylalanine ammonia lyase (PAL) function was assayed according to Chen et al., (2000). The standard Bradford assay was employed to test the protein concentration of root extracts in each sample.

### 2.8. Peroxidase and poly phenol oxidase function assay

Peroxidase (POX) and poly phenol oxidase (PPO) function were assayed according to Liu (2002).

#### 2.8.1. Evaluation of T. harzianum BI for chitinase production

Seven grams of dry wheat bran in a 250 ml Erlenmeyer flask and was supplemented with 3 ml salt solution containing 0.5% $NH_4NO_3$, 0.2% $KH_2PO_4$, 0.1% NaCl, 0.1% $MgSO_4 + 7H_2O$. The initial moisture level in the substrate was adjusted by adding adequate amount of SD/water. The substrate was mixed thoroughly and autoclaved for 20 min at 121 °C (1.5 Atm.) and cooled to room temperature before inoculation. The sterilized solid substrate medium was inoculated with 1ml fungal spore inoculum ($10^6$ spores/ml) under aseptic conditions. The contents were mixed thoroughly and the flasks were placed in an incubator at 30 °C for 10 days and 1 day intervals. Flasks were removed every 24 h and the enzyme extraction and Chitinase assay was done as described below. An adequate amount

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