

Full Length Research Paper

# Biological control of *Meloidogyne javanica* by *Trichoderma harzianum* BI and salicylic acid on Tomato

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In this study, *Trichoderma harzianum* BI was evaluated for its capacity to reduce the incidence and pathogenicity of the root-knot nematode *Meloidogyne javanica* on tomato. Culture filtrates of *T. harzianum* BI at different concentrations, (standard, 1:1, 1:10, and 1:100) were studied. *In vitro* studies revealed that hatching of *M. javanica* eggs were inhibited by the culture filtrates and this inhibition was positively correlated with increase in the concentration of culture filtrates. Parasitism of *M. javanica* eggs by *T. harzianum* BI ranged from 21% in control to 84% in antagonistic fungi. *T. harzianum* BI reduced nematode damage to tomato *in vivo*, too. Treatment of the soil with the antagonistic fungi and salicylic acid clearly improved nematode control when applied jointly or alone. The antagonistic fungi and salicylic acid were further tested for their ability to induce production of defense related enzymes in tomato. Earlier and increased activities of soluble peroxidase (SPOX) was observed in *T. harzianum* BI and salicylic acid treated tomato inoculated with *M. javanica*. Thus, the present study shows that in addition to direct antagonism, induction of defense-related enzymes involved in peroxidase pathway contributed to enhance resistance against invasion of *M. javanica* in tomato.

**Key words:** Biological control, *Meloidogyne javanica*, *Trichoderma harzianum* BI, peroxidase.

## INTRODUCTION

The root-knot nematode *Meloidogyne javanica*, is a common plant parasite in agricultural soil in Iran, it can severely damage growing plants, including tomato, cucumber and melon in the hot weather areas of Iran. *Meloidogyne* spp. is a major pathogen on tomatoes where they cause considerable losses in yields. Nematicides have been used to controlling these pests with remarkable results. However, they have been the main method for controlling of nematode diseases; public concern about nematicides residues in food and environment and also the development of nematicides resistance by pathogens has increased the search for alternative means of controlling disease. 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 (Sahebani and Hadavi, 2008). Biological control of plants decays and vegetables has emerged recently as a promising alternative to the use of synthetic pesticides (Wilson et al., 1993).

Biological control of soil-borne plant pathogens and nematodes by antagonistic microorganisms is a potential non-chemical means of plant disease control (Stirling, 1991). A wide range of bacteria (Hallmann et al., 2001) and fungal agents (Meyer et al., 2001) have been used to reduce a range of plant parasitic nematodes. Some species of *Trichoderma* have been used widely as bio-control agents against soil-borne plant diseases (Whipps, 2001). *Trichoderma* isolates have been used successfully to control of the damage caused by soil-borne pathogens. *Trichoderma* also have been shown to have activity towards root-knot nematode (Sharon et al., 2001). Some *Trichoderma* isolates were reported to do both enhance

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plant growth and reduce root-knot nematode damage (Meyer et al., 2001). However, several attempts have been done to use different species of *Trichoderma* for the control of plant parasitic nematodes but with unsatisfactory levels of control (Dababat et al., 2006). A number of *Trichoderma* isolates are now used commercially for the control of fungal pathogens in the soil.

In this study, the effect of *Trichoderma harzianum* BI as a biocontrol agent for controlling of the root-knot nematode *M. javanica* was investigated the effect of filtrates of *Trichoderma harzianum* BI on Inhibition of egg hatching, direct parasitism of *T. harzianum* BI towards *M. javanica*, and the influence of secondary metabolites on J2 of *M. javanica* mobility and mortality were studied. *In vivo* efficacy of *T. harzianum* BI and salicylic acid against *Meloidogyne javanica* is reported in this paper. The second section of this study was to verify if the induced resistance mechanism is responsible for the capacity of *T. harzianum* BI and salicylic acid, to control root knot disease. The objectives of this section of the study were addressed by monitoring of the activity of SPOX, accepted markers of induced resistance.

## MATERIALS AND METHODS

### Pathogen

Soil and root samples were collected from the rhizosphere of tomato (*Lycopersicon esculentum* Mill.) plants, in the root knot nematodes-infested fields of Varamin. The top 3 to 5 cm of soil layer was removed and about 250 cm<sup>3</sup> of soil and 10 g of feeder roots were collected up to a depth of 30 cm (Santhosh et al., 2005). About 10 soil samples were taken from randomly selected fields in each locality.

The samples were placed in polyethylene bags and brought to the laboratory for the isolation of *Meloidogyne*. Single egg mass was used to establish a population on tomato and cucumber plants. Eggs were extracted from galled tomato roots using 1.5% NaOCl as described by Hussey and Barker (1973), collected on a 25 µm mesh sieve and transferred to a beaker containing tap water. Hatching of second stage juveniles was stimulated by aerating the egg suspension with oxygen in the dark for 7 to 10 days. Freshly hatched second-stage juveniles were separated from the un-hatched eggs using a modified Baermann dish technique. The species of nematode was identified as *M. javanica* according to morphological and morphometrical characters (Eisenback, 1985).

### Biocontrol agent

*Trichoderma harzianum* BI was obtained from plant pathology department, agriculture faculty of Tehran and was cultured on potato dextrose agar (PDA) containing 150 mg l<sup>-1</sup> streptomycin and 150 mg l<sup>-1</sup> of chloramphenicol. The Petri dishes were then placed in an incubator at 25°C. The mycelia and conidia formed were then carefully scraped from the media and suspended in 100 ml tap water. Spores were separated from mycelia by sieving through a 50 µm sieve. The spore suspensions were then adjusted to the desired concentration after counting spore density using a haemocytometer.

### Plant material

Experiments were executed with tomato (*Lycopersicon esculentum* var. Early orbana Y) grown in a controlled-environment cabinet. The air temperature was maintained at 26±2°C.

### Preparation of culture filtrates (CF) of *Trichoderma harzianum* BI and Inhibition of egg hatching

Small blocks of about 5 mm diameter from the pure cultures of *T. harzianum* BI was transferred to potato dextrose broth (PDB) and kept at 25±1°C in a shaking incubator at 60 stroke/min for 10 days. The liquid cultures were passed through a filter paper (Wattman No.1) to obtain spore-free filtrates and designated as standard (Amer et al., 1999). Serial dilutions (1:1, 1:10 and 1:100 V/V) of *T. harzianum* BI culture filtrates in water were prepared subsequently. Eggs of *M. javanica* were surface sterilized with 1.5% NaOCl for 30 s, washed with sterile distilled water three times and transferred to the serial dilutions of culture filtrates of *T. harzianum* BI. There was one isolate of *T. harzianum* BI with 6 replications and each replication contained 45 eggs. The treatments were arranged in completely randomized (CR) design. The data were recorded on egg hatching after ten days of incubation at 28°C. The trials were performed two times and results were combined for statistical analysis (Khattak et al., 2008).

### Effect of *T. harzianum* BI on *M. javanica* eggs

Eggs of *M. javanica* were surface sterilized with 1.5% NaOCl for 15 s, washed with sterile distilled water thrice and transferred to the 10-days old culture of *T. harzianum* BI, 45 eggs per plate. Each treatment was replicated 6 times. Eggs placed on water agar served as control. Eggs were then transferred from *T. harzianum* BI culture into sterile distilled water after 14 days of incubation at 28°C in a dark room. The experiment was repeated, data were taken on egg mortality and the two data were combined for statistical analysis.

### Soil treatment at planting (*in vivo* tests)

Tomato seeds were sown in pots (5 inches in diameter). Seedlings were then propagated for 45 days in the greenhouse in the pots containing sterilized mixture of field soil, leaf compost, and sand at the rate of 1:2: 2:2. In this time, the seedlings (at four-leaf stage) were inoculated with *T. harzianum* BI and salicylic acid, separately and jointly. Each seedling received 20 ml of a liquid suspension of the *T. harzianum* BI containing 10<sup>6</sup> spores and 25 ml of salicylic acid with 5mM concentration. The inoculum was injected 2 cm deep into the rhizosphere using 3 holes made around the stem base with a plastic rod. The absolute controls were treated with distilled water. The pots were then immediately inoculated with a 5 ml sterile distilled water suspension containing 2000 J2 of *M. javanica*. The inoculum was also injected into 3 holes roughly 2-cm deep around the stem base. The experiment consisted of five treatments: 1) Th + Mj, 2) SA + Mj, 3) Th + SA + Mj, 4) only *M. javanica* (positive control), and 5) non-inoculated (negative control). The treated pots were incubated in the greenhouse at 22°C ±5 with 16 h of supplemental artificial light per day. The plants were watered and fertilized with 2 g per liter water to insure proper plant growth.

The experiment was terminated 45 days after fungal and nematode inoculation. Fresh root and shoot weight was measured (Al-Fattah et al., 2007). The roots were removed, washed free of

**Table 1.** Inhibition of *M. javanica* egg hatching in culture filtrates of *T. harzianum* BI.

Treatment	Number of unhatched eggs in culture filtrates			
	1:1	1:10	1:100	Means
<i>T. harzianum</i> BI	41.12%A	26.4%B	16.43%C	27.9%8

Means with different capital letters in the row are significantly different from each other based on duncan Test ( $P \leq 0.05$ ;  $n = 10$ ).

**Table 2.** *In Vitro* parasitism of *Trichoderma harzianum* BI isolates on *Meloidogyne javanica* eggs.

Treatment	Number of infected egg	%Egg infection
Water agar	9.5B	21%B
<i>T. harzianum</i> BI	38A	84%A

Means with different capital letters in the collums are significantly different from each other based on duncan Test ( $P \leq 0.05$ ;  $n = 10$ ).

soil and stained in 0.015% Phloxine B for 20 min to facilitate egg mass counting (Shurtleff and Averre III, 2000). The number of galls, egg masses, eggs and diameter of galls per plant was then determined. The experiment was done in twice. Statistical analysis was done by employing Completely Randomized Experimental Design. ANOVA analysis was performed with critical difference at 5% level of significance.

#### Enzymatic activities determination

Enzymatic activity of SPOX was determined. The treated tomato seedlings similar to the above mentioned methods were used for SPOX. Sodium phosphate buffer (0.1 M, pH 6) was used to extract SPOX. Each treatment consisted of four replicates. Roots from each replicate were sampled separately 1, 2, 3, 4, 5, 6, 7 and 8 days after nematode challenge and ground in a prechilled mortar and pestle. To isolate SPOX, 0.5 g of the sampled roots was ground at 4°C with an appropriate amount of the buffer solution and the homogenate was centrifuged at 4°C for 20 min at 14000 g. The supernatant was transferred to a 1.5 ml vial and stored at -20°C (Madhaiyan et al., 2004).

The SPOX activity measured according to Janda et al. (2003) using guaiacol (Merck, Darmstadt, Germany) as a substrate. Specific enzyme activity was expressed as change in absorbance of the reaction mixture at 470 nm per mg of total protein per min. Protein content in crude extract was determined according to Bradford (1976) with bovine serum albumin as a standard.

## RESULTS

*T. harzianum* BI inhibited of hatching of *M. javanica* eggs and was negatively correlated with the concentration of the culture filtrates of *T. harzianum* BI. The highest inhibition of egg hatching was recorded in 1:1 concentration (41.12%), while the minimum egg hatching inhibition was observed in 1:100 concentration (16.43%) (Table 1). Data in Table 2 showed that *T. harzianum* BI showed significant infection of *M. javanica* eggs. The

percent of unhatched egg in the cultures was 84% compared with 21% in control.

#### Soil treatment at planting (*in vivo* tests)

The treatment of the soil at transplanting with *T. harzianum* BI and *M. javanica* resulted in a reduction of the number of galls, egg masses, eggs and diameter of galls. The significant reduction in gall formation was obtained with *T. harzianum* BI when compared to the control. However, in the experiments, *T. harzianum* BI gave higher levels of control when compared to only *M. javanica* (Table 3). The suppressive effect of the fungi and salicylic acid on egg mass production, which is a measure of nematode development over time, was confirmed for both treatments, when compared to the control (*M. javanica*). *T. harzianum* BI and salicylic acid reduce egg mass, eggs and galls number and diameter of galls (Table 3). Root and shoot weight obviously increased when the tomato plants were inoculated with *T. harzianum* BI and salicylic acid compared to both the absolute and nematode controls (Table 3). The *T. harzianum* BI exists on the surface of the root after 45 days and it was successfully re-isolated from the rhizosphere.

#### Enzymatic activities determination

At the first day after nematode, *T. harzianum* BI and salicylic acid inoculation, soluble peroxidase activity was present at an equal level in extracts from healthy plants and only *M. javanica* treatment but *T. harzianum* BI, salicylic acid and treatments have significant different form other treatments except of salicylic acid treatment. It

**Table 3.** Influence of soil treatment with *T. harzianum* BI and salicylic acid and *Meloidogyne javanica* on gall , egg mass and egg number 45 days after nematode inoculation at the time of transplanting tomato.

Treatment	No. Gall/plant	No. Eggmass/plant	N.Egg/individual egg mass	Diameter of Gall
Th + Mj	55.3 D	34.16 D	408.83 D	1.33 BC
SA + Mj	189.2 B	100 B	310.3 B	1.45 B
Th +SA+Mj	17.2 C	5.66 C	202.17 C	1.09 C
M. javanica	367.2 A	154.83 A	612.33 A	3.51 A
non-inoculated	0	0		0

Means with different capital letters in the collums are significantly different from each other based on duncan Test ( $P \leq 0.05$ ;  $n = 10$ ).

**Table 4.** Time course of change (changes in A(475nm)/min/mg protein) in soluble peroxidase activities in root extracts of tomato treated with sterile water H, *T. harzianum* BI (T), Salicylic acid (SA), and *M. javanica* (N). Enzyme activities were determined as described in the text. Data are average of four replicates.

Treatment	Time after inoculation							
	1	2	3	4	5	6	7	8
T+ SA+N	A44.37g	A65.47f	A95.04d	A138.2a	A119.2b	A102.4c	A79.02e	B42.27g
T+N	B 38.12f	B59.68e	B88 b	B106.07a	C80.38c	B68.02d	C56.4e	B40.37f
SA+N	AB41.07 g	C 47 f	C61 e	D82.61c	B109.5a	A98.85b	B69.4d	A55.3e
N	C 25.1f	D 40.2d	D56.25c	C98.23a	C79.46b	C56.4c	D41.25d	C33.12e
H	C 25.5d	E 27 c	E28.66 c	E33.53a	D33.09a	D30.63b	E27.06c	C32.74a

Means with different capital letters in the collums are significantly different from each other between treatments, and with different minuscule letters in the rows are significantly different from each other between days based on duncan Test ( $P \leq 0.05$ ;  $n = 10$ ).

was increased gradually with *T. harzianum* BI, salicylic acid treatments after this time, however, this increase disappeared, 6 days after nematode inoculation. In control plants inoculated with just the nematode, SPOX activity was reduced significantly from 5 to 8 days after inoculation, compared to activities from the healthy control plants (Table 4).

When the tomato roots were treated with *T. harzianum* BI, salicylic acid and nematode. SPOX activity increased gradually in all the treated roots compared to activities from the infected control plants with nematode. The SPOX activity in treated plants with *T. harzianum* BI in the presence of nematode showed a gradual increase from the initial level of 38 units and reached to a maximum state, 106 units, at 4 days after pathogen inoculation. SPOX activity in the salicylic acid treated plants in the presence of pathogen inoculation was approximately 40% higher than that in the inoculated control roots, at 5 days after inoculation. In all of the day of sampling (except 8 and 1), there were significant difference between *T. harzianum* BI and salicylic acid and other treatments (Table 4).

## DISCUSSION

The significant interaction between *T. harzianum* BI and

concentrations showed that the effectiveness of antagonistic fungi considerably decreased upon dilution. Fungi release toxic metabolites/enzymes into the medium in which they grow (Saksirat and Hoppe, 1991, Bandyopadhyay and Cardwell, 2003). *Trichoderma* spp. has been reported to produce chitinases into the culture (Chet and Baker, 1981), which might help in the inhibition of eggs hatching. Dos Santos et al. (1992) reported *T. harzianum* as an effective egg parasite of *M. incognita*. *T. harzianum* was able to grow on the egg surface and penetrated the egg shell (Saifullah and Thomas, 1996). Saifullah and Thomas; 1996) has used *T. harzianum* against potato cyst nematode (*Globodera rostochiensis*) with excellent results. The present study may indicate that *T. harzianum* is an eggs parasite of root knot nematode. Different species of *Trichoderma* have different modes of penetration (Dumas and Boyonowski, 1992). The variation in egg infection by the *T. harzianum* isolates can be related to genetic variability among the isolates yielding difference in infectivity.

Treatment of the soil with the *T. harzianum* BI or salicylic acid resulted in a reduction in the galls, eggs and egg masses number. Similar results were obtained by other researchers with *Trichoderma*. Sharon et al. (2001) reported that *T. harzianum* reduced galling of root-knot nematode *M. javanica* on tomato plants. Spiegel and Chet (1998) used different *Trichoderma* isolates against

the root-knot nematode *M. javanica*, and the results showed that a decrease in root-galling index as well as eggs per gram of root were achieved when nematode-infested soils were pre-exposed to the *T. harzianum* preparations in short term trials.

In the current study, inoculating the seedlings with *T. harzianum* BI or salicylic acid didn't have a consistent positive effect on fresh root weight, but fresh shoot weight was clearly affected with *T. harzianum* BI or salicylic acid. The results are against those of Sankaranarayanan et al. (2002) who showed that maximum plant height was reached in the non-inoculated control plants followed by those treated with the biocontrol agents. The level of control with *T. harzianum* BI and salicylic acid obtained in this study, using integrated *T. harzianum* BI and salicylic acid against root knot nematode can prove effective for further field development of the antagonistic fungi as a commercial product. The level of biocontrol could be improved significantly, if the *T. harzianum* BI was applied with salicylic acid, simultaneity.

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