Management of the Root-Knot nematode, *Meloidogyne incognita* on Tomato in Egypt

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Abstract: The efficacy of carbofuran at 1 mg a.i./kg soil, *Serratia marcescens* (1 x 10^9 bacterium cells/ml water) at 2 ml of the suspension/kg soil, and three different *Trichoderma harzianum* isolates each separately added at 50 ml./kg soil against the root-knot nematode *Meloidogyne incognita* on two tomato cultivars Super Strain B and Alisa was assessed in the glasshouse. Fresh and dry weight of shoots were higher ($P \leq 0.05$) in nematode-free plants of the two cultivars than both *M. incognita*-infested plants and the above-mentioned treatments. Carbofuran followed by *S. marcescens* and *T. harzianum* generally decreased nematode development and reproduction parameters compared to the untreated control. Although chemical nematicide viz. carbofuran showed a significant effect in increase of growth parameters and in suppression of *Meloidogyne incognita* multiplication, it can be replaced to some extent by microbial antagonists viz. *Serratia marcescens* and *Trichoderma harzianum* isolates to comply with environmental issues confronting the use of chemicals. Our results revealed differences in activities of peroxidase and chitinase related to the above-mentioned treatments on both local (in roots) and systematic (in leaves) levels but late in the growing season as well. [Journal of American Science 2010;6(8):256-262]. (ISSN: 1545-1003).

Keywords: enzymatic induction, *Meloidogyne incognita*, nematode management.

1. Introduction

Root-knot nematode (*Meloidogyne* spp.) infestations on tomato (*Solanum lycopersicum*) are common in Egypt and worldwide and cause severe crop damage especially in light soils (e.g., Abd-Elgawad and Aboul-Eid, 2001; Netscher and Sikora, 1990). Root-knot nematodes can be managed effectively by chemical treatments but many of the nematicides are expensive, pose human and environmental risk or have been withdrawn from use (Greco *et al*., 1992; Abd-Elgawad, 2008). Management of root-knot nematodes with biological control agents has been receiving growing consideration (Abd-Elgawad and Aboul-Eid, 2005, Al-Hazmi *et al.*, 2010). Hence, the present study identified five treatments: a nematicide carbofuran, a bacterium *Serratia marcescens*, and three Egyptian isolates of a fungus *T. harzianum* with an end in view that a clear comparative line could be drawn distinguishing the significance between previously used chemical control with its implied dangers on man and environment and that of biocontrol measure represented by fungi and bacterium aiming at reaching the best policy to be implemented through comparing the five control methods with the untreated growing of two common tomato cultivars; in the presence or absence of the nematode. Since it is fundamental to have prior knowledge of the interaction involved especially in the biocontrol treatments, some enzymatic activities in leaves and roots of tomato plants were assayed.

2. Material and Methods

Pure culture of three Egyptian *Trichoderma harzianum* isolates were provided by The Center of Fungi, Assiut University, Egypt and maintained on Potato Dextrose Agar in Petri plates at 27 ± 5°C. The isolates were designated as f_1, f_3 and f_8 and cultured on healthy sorghum seeds as described by Haseeb *et al* (2005) so that the surface of the seeds was colonized and colony forming units (CFU) reached above to 10^8 CFU/gm culture. Also, eggs were obtained by separation with sodium hypochlorite (0.5%, 1 minute) of *Meloidogyne incognita* egg masses cultured on roots of tomato cv. Strain B plants, and active second stage juveniles (J_2) by egg mass incubation in tap water at 27 ± 1°C in the dark. J_2 were collected after 3 days and concentrated in small volumes of sterilized water by filtering through 1 µm filters (Whatman type) and collecting them after repeated washes. The effect of *T. harzianum* f_1, f_3 and f_8 on egg hatch and J_2 motility of *M. incognita* was tested by in vitro assays similar to the methodology of Suarez *et al.* (2004). Crude culture filtrates (10^6 CFU/ml) of the above-mentioned fungal isolates, compared to sterile deionized distilled water as a check, were added to equal amount (2 ml) of a suspension having about 200 eggs or J_2 of *M. incognita*. Each treatment, had 20 replicates (5-cm Petri dishes), was tested at 25 ± 1°C in the dark and gently shaken every 12 h for one week. Numbers of J_2 that emerged from eggs and immobile J_2 were counted in 1 ml sample from each

dish twice; after 3 and 7 days. Each time, J_2 were transferred in aerated distilled water and then the active nematodes were counted after a day.

Seeds of two tomato cultivars, Super Strain B and Alisa, were germinated in small (3.5-cm diameter) foam wells filled with sterilized peat and a single seedling per well of 11x19-well germination trays and allowed to grow up to the four-true-leaf stage. Afterwards, seedlings were thoroughly washed with tap water and singly transplanted into 140 20-cm-diameter earthen pots filled with a mixture of autoclaved sandy loam soil (sand 68, silt 24% and clay 8%, pH 7.6) and compost (4:1, 2.5 kg/pot) in a greenhouse (26 ± 5 °C and 61% ± 12% Relative Humidity). Plants were periodically watered with Hoagland's nutrient solution. Nematode-treated pots were inoculated with a suspension containing 1000 ± 5 of the J_2 poured in three holes around the plant stem. *T. harzianum*-treated pots received 125 ml/pot of about 10^6 germinated spores/ml of mycelial inoculum gently mixed with soil. In the early morning, ten days after transplanting and immediately after *M. incognita* inoculation, five treatments for each of the two tomato cultivars consisted of adding 4 ml of Nemaless (a commercial suspension of *Serratia marcescens* having 1 x 10^9 bacterium cells/ml water) per pot; mixing carbofuran with soil around plant roots at 1 mg a.i./kg soil; and similarly applying the three fungal isolates separately. Additional two untreated checks for each of the two tomato cultivars included only *M. incognita* inoculation and uninoculated control. The pots were arranged in a randomized complete block design with 20 replicates (pots) and the experiment was repeated once. Data were pooled together for statistical computation.

For enzymatic assays, in each experiment, three seedlings, from each treatment were uprooted 56 days after nematode inoculation. Then the leaves and roots were thoroughly rinsed with tap water, excised from the plants, and separately dried, weighed and kept at -20°C until examined. The roots or leaves were placed in chilled mortars and reduced to powder by grinding after immersion in liquid nitrogen. The powder was homogenized in 1 ml (per 1 g powder) of an acid buffer (84 mM citric acid, 32 mM Na_2HPO_4, and 14 mM 2-mercaptoethanol, pH 2.8). The homogenate was centrifuged at 10,000 rpm for 30 min at 4°C. Total protein content and enzyme activity were determined in the supernatants. Three replicates representing three tomato plants per treatment were used for each test. The method described by Bradford (1976) was adopted to determine protein content. Peroxidase activity was determined spectrophotometrically by measuring the increase in absorbance at 470 nm due to oxidation of guaiacol according to Lee (1973). One unit of enzyme activity was defined as the amount of enzyme that causes 1.0 Optical density (O.D.) min^{-1} change under standard assay conditions. Specific activity was expressed in units mg^{-1} protein. For chitinase assay, the substrate colloidal chitin was prepared from chitin powder according to the method described by Ried and Ogryd-Ziak (1981) and reducing sugar was determined in 1 ml of the supernatant by dinitrosalicylic acid (Monreal and Reese, 1969) using 1 ml of 1% colloidal chitin in 0.05 M citrate phosphate buffer (pH 6.6) in test tubes, 1 ml of enzyme extract was added and mixed by shaking. Chitinase activity was similarly assayed in crude culture filtrates, 6 replicates, of each fungal isolate. Tubes were kept in water both at 37°C for 60 minutes, then cooled and centrifuged before measuring O.D. at 540 nm. Chitinase activity was defined as mM N-acetylglucose amine equivalent released/gram fresh weight tissue/60 minutes.

For nematode-tomato interaction study, the remaining pots were maintained for 60 days after *M. incognita* inoculation; then, the tops of the tested plants were cut off and the roots gently washed free of soil. Fresh and dry weights and lengths of the shoot and root systems as well as nematode galls on roots were counted and rated on a 0—5 scale, known as gall index (Taylor and Sasser, 1978). Figures of egg hatching, J_2 motility, plant growth parameters, protein content and enzyme activities were subjected to analysis of variance and their averages were compared using Duncan's New Multiple Range Test. Final nematode population was counted (Abd-Elgawad and Mohamed, 2006) and divided by 1000 (initial nematode population) to calculate the reproduction factor (RF).

3. Results

The three fungal isolates inhibited ($P \leq 0.05$) egg hatching and J_2 motility of *Meloidogyne incognita* (Table 1). The isolate f_1 had the highest influence. The percentage of J_2 immobility increased with increase of the exposure period. When the J_2 were transferred to water and allowed to recover 13, 11, and 12% of immobile J_2 exposed to f_1, f_3, f_8, respectively were reversible. Differences among such percentages were not significant ($P \leq 0.05$).

Environmental conditions in the greenhouse during the experiment were suitable for both tomato plant growth and nematode infestation and reproduction. So, *Meloidogyne incognita* adversely affected ($P \leq 0.05$) the plant growth parameters of the two cultivars in all treatments compared to uninoculated checks (Tables 2 and 3). This was mainly reflected by fresh and dry weights of plant shoot systems. Apparently, nematode galls

Table 1. Percentage egg hatching and second stage juvenile (J_2) motility of *Meloidogyne incognita* as exposed to three *Trichoderma harzianum* isolates f_1, f_3 and f_8 and distilled water (SDDW) for one week.

Treatment	f_1	f_3	f_8	SDDW
Eggs	20 % c	27% b	31% b	38% a
J_2	59% b	62% b	61% b	76% a

*Average of 20 replicates. Averages in a row sharing a common letter are not significantly ($P \leq 0.05$) different according to Duncan's New Multiple Range Test.

Table 2. Effect of *Trichoderma harzianum*, *Serratia marcescens* and carbofuran on galling scores and reproduction factors of *Meloidogyne incognita* on tomato cv. Super Strain B and plant growth parameters*.

Treatments	Gall Score**	RF+	Fresh weight(gm)		Dry weight(gm)		Length (cm)	
			Shoot	Root	Shoot	Root	Shoot	Root
Nematode (N) only	4.5	1.9	19.6 e	3.1	12.8d	0.8	86.7 c	13.6 b
***T. harzianum* f_1+N**	2.8	0.7	40.3 c	5.2	15.0c	1.3	108.9 b	17.8 a
***T. harzianum* f_3 +N**	3.4	0.9	30.7 d	3.9	14.1cd	0.9	111.0 b	13.4b
***T. harzianum* f_8+N**	3.4	0.8	29.0 d	4.3	13.8cd	1.0	102.5 b	15.1b
Carbofuran +N	2.3	0.6	56.0 b	5.4	17.5b	1.7	116.4ab	13.8b
***S. marcescens* +N**	2.8	0.8	44.4 c	3.5	15.3c	0.9	126.4 a	13.7b
Control	0.0	-------	66.5 a	4.3	21.7a	1.0	127.3 a	18.4a

*Average of 34 replicates. Averages in a column sharing a common letter are not significantly ($P \leq 0.05$) different according to Duncan's New Multiple Range Test.
** Gall index was scored on each plant on a 0-5 basis with 0 = no galls or eggmasses (galls), 1= 1 or 2 galls, 2 = 3-10, 3 = 11-30, 4 = 31-100, and 5 = >100 galls.
+Reproduction factor (RF) = Nematode final population / Nematode initial population.

Table 3. Effect of *Trichoderma harzianum*, *Serratia marcescens* and carbofuran on galling scores and reproduction factors of *Meloidogyne incognita* on tomato cv. Alisa and plant growth parameters*. 0.05) different according to Duncan's New Multiple Range Test.

Treatments	Gall Score**	RF+	Fresh weight(gm)		Dry weight(gm)		Length (cm)	
			Shoot	Root	Shoot	Root	Shoot	Root
Nematode (N) only	3.9	1.4	31.0 d	5.1	13.6 d	2.7	83.6 c	15.5c
***T. harzianum* f_1+N**	2.9	0.8	49.9 bc	7.3	25.1bc	3.7	107ab	20.8b
***T. harzianum* f_3 +N**	3.0	0.9	48.0 c	7.2	24.8bc	3.5	104.9b	21.3b
***T. harzianum* f_8+N**	3.1	1.0	45.8 c	6.9	21.4 c	3.4	99.5b	21.8b
Carbofuran + N	2.2	0.4	58.6 b	5.4	30.8 b	2.5	115 a	19.3b
***S. marcescens* +N**	2.9	0.9	52.8 bc	5.6	27.2bc	2.6	116.2a	26.5a
Control	0.0	-----	74.5 a	10.3	41.7 a	5.5	123.9a	27.1a

*Average of 34 replicates. Averages in a column sharing a common letter are not significantly ($P \leq$
** Gall index was scored on each plant on a 0-5 basis with 0 = no galls or eggmasses (galls), 1= 1 or 2 galls, 2 = 3-10, 3 = 11-30, 4 = 31-100, and 5 = >100 galls.
+Reproduction factor (RF) = Nematode final population / Nematode initial population.

formed on infected roots could confuse their weights as a dependable growth parameter for comparison. Nematode-gall index, of untreated inoculated plants, was higher on roots of tomato cv. Super Strain B than cv. Alisa indicating that the first cultivar is probably more susceptible to the tested nematode population. Generally, maximum increase in all the growth parameters of the two tomato cultivars was found in carbofuran treated plants followed by *S. marcescens*, *T. harzianum* f_1, *T. harzianum* f_3, *T. harzianum* f_8 treated plants as compared to untreated inoculated plants. No galls were found on roots of the control plants demonstrating no nematode contamination. Nematode final population did not increase over their initial populations in all treatments but the reproduction factor exceeded one in untreated inoculated plants of the two cultivars.

Protein content, chitinase and peroxidase activities in leaves and chitinase and peroxidase activities in roots of the two tomato cultivars are shown in tables (4 and 5), respectively. Protein content in plant leaves of tomato cvs Super Strain B and Alisa ranged 4.22 - 19.31and 4.94 - 6.84 mg/gm fresh weight, respectively. It was significantly ($P \leq 0.5$) higher only in *T. harzianum* f_8-treated plants than in other treatments and controls of the cultivar Super Strain B but no significant difference was detected concerning protein contents of tomato cv. Alisa plants. Chitinase activities in plant leaves of cv. Super Strain B-treated plants were generally higher than those of Alisa plants. *T. harzianum* f_1-treated plants demonstrated the highest ($P \leq 0.05$) activity. Uninoculated Super Strain B plants had the lowest ($P \leq 0.05$) level of chitinase activity. No significant ($P \leq 0.05$) difference in chitinase activity was found among treatments of Alisa cultivar. Also, chitinases assayed in the crude culture filtrates of the three *T. harzianum* isolates f_1 f_2 and f_8 were 1.82, 0.78 and 1.98 mM N-acetylglucose amine equivalent released / gram filtrate / 60 minutes, respectively. Differences in peroxidase activities in leaves of the two tomato cultivars at 56 days after nematode inoculation were not significant. Yet, such a difference was significantly ($P \leq 0.05$) less in the roots of *T. harzianum* f_1+nematode-treated cv. Alisa plants than the control in the presence or absence of the nematode (Table 4). Chitinase in the roots of *T. harzianum* f_1+ nematode-treated cv. Alisa plants was significantly ($P \leq 0.05$) less than those of untreated inoculated plants.

Table 4. Protein content, chitinase and peroxidase activities in leaves of two tomato cultivars grown in soil treated with *Trichoderma harzianum*, *Serratia marcescens* or carbofuran and/or *Meloidogyne incognit* as compared to untreated plants*.

Treatments	Tomato cv. Super Strain B			Tomato cv. Alisa		
	Protein	Chitinase	Peroxidase	Protein	Chitinase	Peroxidase
Nematode (N) only	5.91 b	10.12 bc	0.15	5.75	7.94	0.106
***T. harzianum* f_1+N**	4.56 b	25.74 a	0.19	6.27	5.63	0.043
***T. harzianum* f_3 +N**	7.96 b	11.0 bc	0.17	4.94	7.34	0.069
***T. harzianum* f_8+N**	19.31a	12.25 bc	0.16	6.28	6.30	0.060
Carbofuran + N	8.07 b	10.12 bc	0.19	6.57	8.30	0.087
***S. marcescens* +N**	8.53 b	15.31 b	0.24	5.80	11.93	0.082
Control	4.22 b	7.83 c	0.25	6.84	9.44	0.169

*Average of six replicates. Averages in a column sharing a common letter are not significantly ($P \leq 0.05$) different according to Duncan's New Multiple Range Test.

Table 5. Chitinase and peroxidase activities in roots of two tomato cultivars grown in soil treated with *Trichoderma harzianum* f_1 and *Meloidogyne incognit* as compared to controls in the presence or absence of the nematode *.

Treatments	Tomato cv. Super Strain B		Tomato cv. Alisa	
	Chitinase	Peroxidase	Chitinase	Peroxidase
Nematode (N) only	1.90	0.042	2.41 a	0.25 a
***T. harzianum* f_1+N**	1.92	0.037	1.23 b	0.04 b
Control	1.31	0.038	1.91 ab	0.17 a

*Average of six replicates. Averages in a column sharing a common letter are not significantly ($P \leq 0.05$) different according to Duncan's New Multiple Range Test.

4. Discussion

Although chemical nematicides, like carbofuran, demonstrated high effectiveness ($P \leq 0.05$) against the root-knot nematodes (Tables 2 and 3), biological control of plant-parasitic nematodes by microorganisms such as *Serratia marcescens* and *Trichoderma harzianum* has been considered a more natural and environmentally acceptable alternative to such chemicals (Suarez *et al*., 2004, Abd-Elgawad and Mohamed, 2006). Thus, the overall goal of such biocontrol agents is the identification and deployment of highly effective strain(s) against several plant pathogenic fungi and/or nematode pests before their development into registered, ready-for-sale plant protection products. Admittedly, strains of *T. harzianum* are able to antagonize numerous phytopathogenic fungi (e.g., Harman, 2000, Hajieghrari *et al*., 2008) and suppress *Meloidogyne* spp. as the most economically important group of phytonematodes worldwide (Rao *et al*., 1996, Sharon *et al*., 2001, Spiegel and Chet, 1998, Windham *et al*., 1993, Suarez *et al*., 2004). Commercial products of *T. harzianum* are available (Harman, 2000) but local ones may be more adaptive and less expensive without any risk to Egyptian fauna and flora than imported strains. So, the relatively high efficacy demonstrated by *T. harzianum* f_1 (Tables 1-5) may nominate it for further experimentations and development. Increase in the efficacy of the fungi appears possible when such biocontrol agents are integrated with organic amendments such as oil cakes (Parvatha Reddy *et al*., 1996) and wheat bran-peat preparations (Sharon *et al*., 2001). Eventually, although chemical nematicide viz. carbofuran showed a significant effect in increase of growth parameters and in suppression of *Meloidogyne incognita* multiplication, it can be replaced to some extent by microbial antagonists viz. *Serratia marcescens* and *Trichoderma harzianum* isolates to comply with environmental issues confronting the use of chemicals.

The mechanisms by which strains of *T. harzianum* function against phytopathogenic fungi are mycoparasitism, antibiosis, competition for nutrients or space, tolerance to stress through enhanced root and plant development, induced resistance, solubilization and sequestration of inorganic nutrients, inactivation of the pathogen's enzymes or/and enzymatic hydrolysis (Sivan and Chet, 1992, Harman, 2000). All mechanisms, except competition, can potentially be involved in the nematode biocontrol process (Sharon *et al*., 2001). Understanding the possible mechanisms of this fungal activity against nematodes could lead to the development of improved biocontrol application methods and selection of active isolates. Direct interactions between *T. harzianum* and the potato cyst nematode *Globodera rostochiensis* were demonstrated in vitro by Saifullah and Thomas (1996). The fungus penetrated the cysts and the eggs in those cysts, resulting in larval death. Two mechanisms were suggested against the root-knot nematodes: effect of metabolites produced by the fungus in the soil and direct parasitism by the antagonist (Sharon *et al*., 2001). Our visible observations by the light microscopy (Table 1) support such suggestion since many non-viable and distorted eggs were found in the fungal treated dishes.

In leaves of tomato cvs Super Strain B and Alisa, significant ($P \leq 0.05$) differences were observed among treatments only in the protein content and chitinase activities of the first cultivar (Table 4). So, such differences could be cultivar specific but also could be the result of plant-microorganism interaction. Also, differences in peroxidase and chitinase activities were pronounced in roots of only Alisa cultivar (Table 5). Yet, peroxidase activities had the highest level in roots of another susceptible tomato cultivar 56 days after *M. incognita* inoculation as well (Mohamed *et al*., 2003).

It is well known that defence reactions in Solanaceae depend primarily on the type of elicitor, on the plant genotype/species, or on the type (compatible or incompatible) of interaction (Desender *et al*., 2007). Indeed, in tomato roots infected with root-knot nematodes, genes with homology to several known plant defense genes including peroxidase and chitinase are induced locally within 12 h of inoculation (Williamson and Hussey, 1996) but systematically when invaded by *T. harzianum* (Yedidia *et al*., 1999). Defence gene transcription or enzyme activity is, most of the time, delayed and lower in compatible (susceptible plant) than in incompatible (resistant plant) interactions. Our results revealed such enzyme activities on both local (in roots) and systematic (in leaves) levels but late in the growing season as well (Tables 4 and 5). Yet, the comparison of reaction patterns highlighted by Desender *et al*. (2007) suggested that these patterns are as a rule specific to each plant genotype/elicitor pair, irrespective of the compatibility/incompatibility status of the interaction. Hence, our present study characterized such patterns of two genotype/elicitor pairs (Tables 2-5). Additional studies are needed to clarify the interaction of *Meloidogyne* spp. with *T. harzianum* as a biocontrol agent in terms of the physiological roles of enzyme activities in response to nematode attack and fungal colonization.

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