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Antagonistic effects of some indigenous isolates of *Trichoderma* spp. against *Meloidogyne javanica*

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Abstract

Two studies, *in vitro* and in the green house, were conducted to determine the efficacy of some Saudi *Trichoderma* isolates against *Meloidogyne javanica*. The isolates included: *Trichoderma hamatum* (one isolate), *T. viride* (one isolate), *T. asperellum* (three isolates), *T. atroviride* (one isolate) and *T. harzianum* (two isolates). *In vitro*, results showed that all eight tested isolates and their culture filtrates were effective at different levels, in egg hatch inhibition and mortality of juvenile (J_2). The mortality of J_2 and un-hatching of eggs increased as the concentrations of the culture filtrates increased. In general, *T. harzianum* (isolate No.27), *T. hamatum* (isolate No.5) and *T. viride* (isolate No.8) were the most effective among all tested isolates. Therefore, these three isolates were further evaluated in the greenhouse test on tomato against *M. javanica*. The three tested isolates inhibited the reproduction, root galling and number of juveniles of *M. javanica*. The *T. harzianum* (isolate No.27) was the most effective isolate.

Keywords: Biocontrol, egg hatch, juvenile mortality, root-knot nematodes, tomato.

The nematophagous fungi have attracted an increasing attention as important biological control means for nematodes (Kerry, 1987; Jansson & Lòpez-Liorca, 2004; Sharon *et al*., 2011; Hallman *et al*., 2009). In the natural ecosystem, these fungi are important in regulating nematodes parasite of plants, due to their antagonistic, parasitic, pathogenic activities, or by producing toxic metabolites (Viane *et al*., 2006). They can parasitize nematodes directly or indirectly by secretion of nematicidal compounds that may affect nematode (Lòpez-Liorca *et al*., 2008; Yang & Zhang, 2014).

The genus *Trichoderma* is one of the fast-growing fungi, and is widely distributed in soil. Its different species have attracted much attention as biocontrol agents of nematodes (Sharon *et al*., 2011). Besides their parasitic activities, the different species of *Trichoderma* produce some nematicidal compounds such as acetic acids (Sharon *et al*., 2011). The *Trichoderma* species showed different levels of success against root-knot nematodes (Meyer *et al*., 2001; Sharon *et al*., 2001, 2007; Spiegel *et al*., 2007; Suarez *et al*., 2004).

In *Trichoderma* parasitism is an important mode of action against nematodes (Sharon *et al*., 2011). The second mechanism involved the production of different enzymes, toxins and secondary metabolites (Sharon *et al*., 2011; Suarez *et al*., 2004; Howell, 2003). Several species of *Trichoderma* are very promising biocontrol agents and have potentials to suppress population of nematodes (Sharon *et al*., 2001).

The objectives of this study include: 1) *in vitro* effects of eight Saudi isolates of five *Trichoderma* species (Table 1) and their filtrates J_2 mortality and egg hatching of *Meloidogyne javanica* and; 2) the relative efficacy of the three most effective isolates of *Trichoderma* spp., found in the *in vitro* tests, namely: *T. harzianum*, *T. hamatum* (Bon.) and *T. viride* as biocontrol agents against *M. javanica* on tomato in the greenhouse.

Materials and Methods

***Meloidogyne javanica*:** Whenever needed, egg-masses of *M. javanica* were hand-picked from a greenhouse pure culture of the nematode on tomato plants (cv. Sultana-7). The egg-masses were surface sterilized, and eggs were then extracted (Hussey & Barker, 1973) and washed in sterilized distilled water. Eggs were collected in a small volume of sterilized distilled water by passing over 38 µm sieve. These eggs were used for the egg hatch and greenhouse tests. For the juvenile mortality test, the extracted eggs were placed on hatching cups in sterilized distilled water to hatch to second-stage juveniles (J_2). Then, juveniles (J_2) hatched from these eggs were used in mortality test.

Isolates of *Trichoderma*: The eight Saudi isolates of five *Trichoderma* species namely: *T. hamatum* (isolate No. 05), *T. harzianum* (isolate No. 23 and 27), *T. viride* (isolate No. 08), *T. atroviride* (isolate No. 18) and *T. asperellum* (isolate No. 01, 10 and 16) were obtained from the mycology unit (Prof. Younes Y. Molan), laboratory of fungal and bacterial diseases of the Department. These fungi were originally isolated from Riyadh region, Saudi Arabia using the method described by Elad & Chet (1983). The fungi isolates were identified to species level by ITS1 and ITS2 of ribosomal DNA (Maymon *et al*., 2004; Hermosa *et al.*, 2000).

The eight fungal isolates were, then, cultured on PDA in Petri dishes in an incubator at 28°C for a week. For preparation of culture filtrate of each isolate, potato dextrose broth (PDB) (150 ml in 250 ml conical flasks) was prepared and sterilized. After that flasks were inoculated with 5 mm diameter block of the pure culture of the designated *Trichoderma* isolate grown on PDA (Qureshi *et al*., 2012). The inoculated flasks were, then, left for 3 weeks at room temperature. Then, the hyphal mats were removed from the liquid cultures (primary filtration) by passing the culture through Whatman No. 1 filter paper to avoid the mycelium (Qureshi *et al*., 2012). Then, a secondary filtration was done by Millipore using 0.45 µm membrane filter for removal of spores. The obtained filtrate served as a standard stock filtrate for screening nematicidal activity of different concentrations of the culture filtrate of each isolate.

A. *In vitro* study

Direct effects of *Trichoderma* isolates on egg hatch and juvenile mortality: Water agar (WA) in Petri plates were used in the two parasitism tests. The center of each Petri plate was inoculated with a block of a one week old culture (on PDA) of the designated *Trichoderma* isolate. Non-inoculated water agar plates served as controls.

In the egg hatch test, 65 eggs of *M. javanica* was added in the Petri plates containing the designated fungal isolate. Eggs were also placed on fungus-free water agar plates as controls. Each treatment (Table 1) comprised of four replications. Treatments were placed in a completely randomized design (CRD) at room temperature (24±2°C) (Jamshidnejad *et al*., 2013). Two weeks later, data on egg hatch was recorded. The hatched juveniles (J_2) per Petri plate were counted and ratio of hatch inhibition was determined.

In the test of the juvenile mortality, 65 fresh J_2 (in one ml water) were added to water agar Petri plates containing the designated fungal isolate (8 isolates). All inoculated Petri plates were left on a Table at room temperature (24±2 °C) (Jamshidnejad *et al*., 2013).The test comprised of 4 replications and nine treatments (Table 1) in a completely randomized design. After 72 h, the active J_2 (moving when touched) were counted. Juvenile's mortality was calculated and recorded.

Effect of culture filtrate of *Trichoderma* isolates on egg hatching and juvenile mortality: The test on egg hatch was conducted under sterile conditions. Autoclaved small glass vials (size 17 mm diameter × 57 mm height) were used. Culture filtrate of each isolate was used at 25, 50 and 75% concentrations of the standard stock filtrate (Table 2). For 25% treatment, 1 ml of stock filtrate was added in 3 ml sterile water containing 165 sterile

eggs in each vial to give a final concentration of 25%. Similarly, the 50% and 75% concentrations treatments were made. The treatment only with sterile water was used as a control.

Each treatment comprised of four repeats. The vials were, then, left at room temperature (24±2℃) in a completely randomized design. Seven days later, the hatched juveniles (J_2) were counted, and the percentages of hatch inhibition were calculated.

The test on juvenile mortality was conducted in a similar procedure as described above for the effect of culture filtrate on egg hatch, except that J_2 were used instead of eggs. After 72 h, one ml of the suspension from each vial was transferred to a counting chamber and mortality of juveniles were recorded. The juveniles which were inactivate, by touching with a small needle and were still immobile and straight, were considered as dead. Percent of mortality was calculated.

B. Greenhouse study

Effect of *Trichoderma* isolates against *Meloidogyne javanica* on tomato in green house: In the greenhouse (24±2℃) tomato (cv. Sultana-7) were used in 15 cm diameter containing 1500 g sterilized soil mixture (sand, clay and peat moss) in 2:1:1, respectively.

Based on the results obtained from our *in vitro* tests, the three most effective isolates were; *T. harzianum* (isolate No. 27), *T. viride* (isolate No.08) and *T. hamatum* (isolate No. 05). The selective *Trichoderma* isolates were cultured on PDA in an incubator at 24℃ for almost two weeks. The conidia were collected and conidial concentration 1×10^8 conidia (at rate of per gram soil) was mixed in each pot, following by mixing of 10,000 *M. javanica* eggs per pot. Then tomato seedlings were transplanted in the pots which are already infested with nematodes and fungi. Untreated seedlings, *Trichoderma* only and nematodes only infested seedlings were served as control. The nematicide Carbofuran (Furadan® 10 G) (0.2 g/pot) was used as a chemical control treatment to compare it with biocontrol agents (*Trichoderma* species). The treatments (Table 4) were arranged in a complete randomized design on a green house bench. The experiment was finished after 55 days. Plant root and shoot fresh weight, root galling, number of eggs and egg-masses were recorded. The nematode final population density and reproduction factor were calculated by extracting the second-stage-juvenile (J_2) from soil.

Data analysis

Data from all *in vitro* and greenhouse tests were analyzed according to analysis of variance (ANOVA). The difference in treatment means were analyzed at $P \leqslant 0.05$ by least significant differences (LSD) using SAS (SAS, 2013).

Results

A. *In vitro* study

Direct effects of *Trichoderma* isolates on egg hatch and juvenile mortality: Egg hatch was decreased ($P \leqslant 0.05$) by all eight isolates in comparison to control treatment (Table 1). The highest inhibition of hatch was achieved by *T. harzianum* (isolate No. 27), followed by *T. hamatum* (isolate No. 05) and *T. asperellum* (isolate No. 01). The effects varied ($P \leqslant 0.05$) among all the isolates, but always higher than control (Table 1).

The test on juvenile (J_2) mortality showed that only three isolates namely: *Trichoderma harzianum* (isolate No. 27), *T. hamatum* (isolate No.05) and *T. asperellum* (isolate No. 01) caused more than 50% of juvenile mortality (Table 1). Although, the other five isolates caused less than 50% mortality, they caused higher ($P \leqslant 0.05$) mortality as compared to control treatment.

Table 1. Effect of *Trichoderma* species and isolates on egg hatch and juvenile mortality of *Meloidogyne javanica.*

Fungus	Eggs*		Juveniles (J_2)*	
	Un-hatched eggs	% Inhibition	Dead J_2	% Mortality
T. harzianum (isolate No. 27)	38 a	58	42 a	65
T. hamatum (isolate No. 05)	34 b	52	40 b	61
T. asperellum (isolate No. 01)	27 c	42	35 c	53
T. viride (isolate No. 08)	24 d	37	31d	48
T. asperellum (isolate No. 16)	19 e	29	30 d	46
T. asperellum (isolate No. 10)	14 f	22	25 e	23
T. atroviride (isolate No. 18)	12 f	19	15 f	38
T. harzianum (isolate No. 23)	7 g	11	11 g	18
Control	4 h	7	7 h	10

Values are means of four replicates. Means, in each column, followed by same letter are not significantly different ($P \leqslant 0.05$): *Out of 65 eggs and 65 juveniles. Recording of egg hatch was after 2 weeks, and of juvenile mortality after 7 days.

Effects of culture filtrates of *Trichoderma* isolates on egg hatching and juvenile mortality: Almost culture filtrates from each isolates inhibited ($P \leqslant 0.05$) egg hatch at all concentrations, compared to the control (Table 2). Inhibition varied with the fungal isolate and concentration but, generally, increased as the filtrate concentration was increased (Table 2). *Trichoderma viride* (isolate No. 08), *T. hamatum* (isolate No.05) and *T. harzianum* (isolate No. 27) caused more than 90% inhibition of egg hatch at 75% concentration (Table 2).

Culture filtrates of all tested isolates at all concentrations increased the mortality of the juveniles (J_2) compared to the control (Table 3). Mortality varied with the fungal isolate and the concentration of the culture filtrate (Table 3). *Trichoderma hamatum* (isolate No. 05), *T. viride* (isolate No. 08) and *T. harzianum* (isolate No. 27) caused more than 90% mortality of the juveniles at 75% concentrations (Table 3).

B. Greenhouse study

Effect of *Trichoderma* isolates against *Meloidogyne javanica* on tomato in greenhouse: Both *Trichoderma harzianum* (isolate No. 27) and *T. hamatum* (isolate No. 05) increased ($P \leq 0.05$) the total plant weight (Table 4). However, weight was not increased in case of *T. viride* (isolate No.08). But all isolates of *Trichoderma* was effective in decreasing ($P \leq 0.05$) the number of root galls as compared to *M. javanica* only inoculated control treatment plants (Table 4). But, nematicide Carbofuran was more effective in reducing root galling ($P \leq 0.05$) than the fungal isolates and effect of these isolates varied with respect to each other.

While all *Trichoderma* species were effective in reducing the number of eggs, egg-masses and J_2 ($P \leq 0.05$) (Table 5), but the rate of suppression of nematode varied with species; *T. harzianum* was the most effective species (Table 5). As it was expected in case of Carbofuran, nematode reproduction was suppressed significantly ($P \leq 0.05$).

Table 2. Effects of culture filtrate, at different concentrations, of *Trichoderma* species and isolates on egg hatch of *M. javanica*

Fungus	Concentration of culture filtrate					
	25%		50%		75%	
	Un-hatched eggs*	% Hatch inhibition	Un-hatched eggs*	% Hatch inhibition	Un-hatched eggs*	% Hatch inhibition
T. viride (isolate No. 08)	112 b	68 b	145a	88 a	159 a	96a
T. hamatum (isolate No. 05)	131 a	79 a	144a	87 a	155 a	94a
T. harzianum (isolate No. 27)	86 c	54 c	122 b	74b	150 a	91 a
T. harzianum (isolate No. 23)	74 c	44 c	97 c	59 c	122 b	74 b
T. atroviride (isolate No.18)	69 c	42 c	108 bc	65 bc	116 b	71 b
T. asperellum (isolate No. 10)	69 c	42 c	83 d	50 d	114 b	69 b
T. asperellum (isolate No. 01)	32 d	14 d	50 e	30 e	87 c	53 c
T. asperellum (isolate No. 16)	41 d	19 d	38 e	23e	67 d	40 d
Control (water)	13 e	8 e	13 f	8 f	13 e	8 e

*Values are means of four replicates. Means, in each column, followed by the same letters are not significantly different ($P \leqslant 0.05$):*Out of 165 eggs. Recording of egg hatch was after 7 days.

Table 3. Effect of culture filtrate of *Trichoderma* species and isolates on mortality of the second stage-juveniles (J_2) of *Meloidogyne javanica.*

Fungus	Concentration of culture filtrate					
	25%		50%		75%	
	Dead J_2*	% Mortality	Dead J_2*	% Mortality	Dead J_2*	% Mortality
T. hamatum (isolate No. 05)	134 a	81a	143 ab	86 ab	160a	97 a
T. viride (isolate No. 08)	61 c	37 c	145a	88 a	159 a	96 a
T. harzianum (isolate No. 27)	84 b	51 b	142b	86 b	157 a	95 a
T. harzianum (isolate No. 23)	33 f	20 f	116 c	71 c	146 b	89 b
T. atroviride (isolate No. 18)	20 g	12 g	92 d	56 d	118 c	71 c
T. asperellum (isolate No. 16)	34 f	20f	56 f	34 f	103 d	63 d
T. asperellum (isolate No. 10)	39 e	23e	52 g	32 g	101 de	61de
T. asperellum (isolate No. 01)	58 d	35d	81 e	49 e	100 e	61e
Control (water)	13 h	8 h	13 h	8 h	13 f	8 f

Values are means of four replicates. Means, in each column, followed by same letters are not significantly different ($P \leqslant 0.05$):*Out of 165 juveniles. Recording juveniles was after 72 hrs.

Table 4. Effects of three *Trichoderma* species on root galling and plant growth of tomato plants (cv. Sultana-7) infected with *Meloidogyne javanica*.

Treatments	TPW*	Galls/g root	Galls/root system	% Reduction (based on root system)
Meloidogyne javanica	46cd	129a	901a	--
M. javanica + *T. hamatum* (isolate No. 05)	68a	95b	731b	19
M. javanica + *T. viride* (isolate No. 08)	50cd	87c	597c	34
M. javanica + *T. harzianum* (isolate No. 27)	68a	79d	510d	43
M. javanica + carbofuran	44d	54e	268e	70
Untreated seedlings	57c	-	-	-

Values are means of four replicates. Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$); *TPW: Total plant fresh weight (g).

Table 5. Effects of three *Trichoderma* species on reproduction of *Meloidogyne javanica* on tomato (cv. Sultana-7).

Treatment	Eggs/g root	Egg-masses/g root	J_2/100 g soil	*Rf
M. javanica only	18864 a	64 a	218a	12a
M. javanica + *T. hamatum* (isolate No. 05)	10426 b	32 b	185b	8 b
M. javanica + *T. viride* (isolate No. 08)	9458c	28 c	156c	7 c
M. javanica + *T. harzianum* (isolate No. 27)	7373 d	20 d	131d	5 d
M. javanica + carbofuran	3519e	14 e	112e	2 e

Data are means of four replicates. Means, in each column, followed by the same letter are not significantly different ($P \leq 0.05$):*Rf: Reproduction factor = Initial inoculum density (Pf) /Final inoculum density (Pi).

Discussion

The results of the *in vitro* and greenhouse tests showed that all of the tested isolates and their culture filtrates were found to be effective against *M. javanica*. The egg un-hatching and J_2 mortality increased as the culture filtrates increased. Although our all isolates were effective in reducing egg hatch and juvenile viabilities, there were some variations in their efficacy. This could be related to genetic variability among the isolates leading to differences in infectivity. Generally, *T. harzianum* (isolate No. 27), *T. hamatum* (isolate No. 05) and *T. viride* (isolate No. 08) were the most effective among all tested isolates. The culture filtrates of these three isolates caused more than 90% mortality to J_2. Our results support previous reports on the efficacy of *T. harzianum* (Siddiqui & Shaukat, 2004; Sharon *et al*., 2001; Jamshidnejad *et al*., 2013), *T. hamatum* and *T. viride* (Liu *et al*., 2007; Sharon *et al*., 2007). Both *in vitro* tests indicate that direct parasitism and some nematicidal metabolites and enzymes are involved. Different mechanisms were described

for the activities of *Trichoderma* spp. against plant-parasitic nematodes, including parasitism and secreting the nematicidal compounds which affect nematode survival (Liu *et al*., 2007; Sharon *et al*., 2001). Direct parasitism of *T. harzianum* (T-203) on *M. javanica in vitro* has been reported (Sharon *et al*., 2001). Protease enzyme was also secreted by the fungus. *Trichoderma* can produce chitinases in the culture filtrate (Sharon *et al*., 2001), which might help in the inhibition of egg hatch. We selected the most effective three isolates in the *in vitro* study (*T. harzianum* isolate No. 27, *T. hamatum* isolate No. 05 and *T. viride* isolate No. 08) for further evaluation, in greenhouse.

The results of the green house study show that all species have the ability to suppress the reproduction of nematode, root galling ratio, as well as, can improve the growth of plants infected with the nematodes. However, its ability depends upon type of fungal species; generally speaking, *T. harzianum* was the most effective species. *T. harzianum* is one of the most studied species against *M. javanica*, and always found more effective as compared to the other *Trichoderma* species. This green house study supports previous results related to the effectiveness of various *Trichoderma* species against the root-knot nematodes on vegetable crops (Siddiqui *et al*., 2001; Windham *et al*., 1989; Sharon *et al*., 2001; Meyer *et al*., 2001, Tariq Javeed & Al-Hazmi, 2015).

All tested species were able to suppress the *M. javanica* reproduction on tomato. Greatest (43.38%) suppression was achieved by *T. harzianum* (isolate No. 27) but in comparison to nematicide Carbofuran was much more effective as it suppressed the reproduction up to 70%. It is always understood that nematicides provide very effective and faster result by suppression of nematodes reproduction rates (Kerry, 2000; Haydock *et al*., 2006). But, these nematicides are not environment friendly and have many drawbacks; thus, alternative control measures like biocontrol agents, are needed for managing nematodes; which should be safer to environment. The use of *Trichoderma* species have some benefits as they boost up the plant growth and induce resistance in tomato as described previously (Inbar *et al*., 1994; Yedidia *et al*., 1999). Two best species viz., *Trichoderma harzianum* (isolate No. 27) and *T. viridi* were further tested on basis of different inoculum densities (Al-Hazmi & Tariq Javeed, 2016), they give the same results for inoculum density 10^8 conidia per gram of soil which was used in this experiment. This indicates that these indigenous isolates have some potential as biological control agents against *M. javanica* and other important root-knot nematode species in our vegetable system.

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