African Journal of Biotechnology Vol. 11(96), pp. 16247-16252, 29 November, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.233
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Biological control of *Meloidogyne incognita* by *Trichoderma harzianum* and *Serratia marcescens* and their related enzymatic changes in tomato roots

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Accepted 2 March, 2012

Biological control against the root-knot nematode, *Meloidogyne incognita* was proven to occur in tomato, *Solanum lycopersicom*, soil-drenched with different isolates of *Trichoderma harzianum* and a commercial suspension of *Serratia marcescens* (Nemaless). The potential of such biocontrol agents to trigger plant defense response was discussed. Nematode reproduction in the presence of such possibly induced systemic resistance (ISR) elicitors was compared with that occurring on untreated plants and treated plants with the carbofuran nematicide. Dosages used were for carbofuran (1 mg ai/kg soil) and for *S. marcescens* (1×10^9 bacterium cells/ml water) 2 ml suspension/kg soil; three different *T. harzianum* isolates (f_1, f_3 and f_8) were separately added at 50×10^8 CFU/kg soil. The possible ISR elicitors were tested on two tomato cultivars (Super Strain B and Alisa), which were inoculated with active juveniles (J_2) of *M. incognita*, and plants were kept in a glasshouse. Indices of plant fitness (PFs) resulting from each treatment, which took into account various growth parameters were also determined. Carbofuran followed by *S. marcescens* and *T. harzianum* significantly decreased ($P \leq 0.05$) nematode development and reproduction when compared with the untreated controls. PF of cv. Alisa was higher than that of Super Strain B, and *M. incognita* reproduced better on the latter cultivar in all treatments. Polyphenol oxidase (PPO) and β-1,3-glucanase (GLUC) activities were detected in the roots of inoculated and uninoculated control tomato plants. Similar tests were carried out on inoculated plants treated with such ISR elicitors to search for possible enzyme activity changes as a result of resistance induction. Nematode infection did not cause any significant changes in GLUC activity, whilst PPO activity was enhanced in inoculated with respect to uninoculated roots. Treatments with ISR elicitors and carbofuran did not significantly change GLUC activity in both inoculated plants and uninoculated controls. While in the presence of the ISR elicitors, generally, PPO activity did not increase as a result of nematode infestation.

Key words: Enzymatic induction, root-knot nematode, nematode management, *Serratia marcescens*, *Trichoderma harzianum*, biological control, carbofuran, nematicide, polyphenol oxidase, β-1,3-glucanase, *Solanum lycopersicum.*

INTRODUCTION

Root-knot nematodes (RKNs), *Meloidogyne* spp., are considered the most damaging nematode group in the world as they cause severe yield losses to many economically important plant species in subtropical and tropical regions (Luc et al., 2005). Their infestations on tomato (*Solanum lycopersicum* L.) are common in Egypt and worldwide; causing high crop damage especially in light soils (Kheir and Osman, 1977; Netscher and Sikora, 1990).

Root-knot nematodes can be managed effectively by chemical treatments but many of the nematicides are expensive or cause human and environmental risk (Kheir

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and Osman, 1977; Greco et al.,1992). Nematode resistant tomato, especially the hybrid cultivars as a non-chemical strategy may be used under protected cultivation in Egypt but their costly seeds and the emergence of virulent nematode forms limit such a strategy in open fields. Management of root-knot nematodes with biological control agents or with nonchemical approach has received more attention (Kheir and Osman, 1977; Al-Hazmi et al., 2010). Hence, the present study compares five treatments: a nematicide (carbofuran), a bacterium (*Serratia marcescens* Bizio), and three Egyptian isolates of a fungus (*Trichoderma harzianum* Rifai) on two common tomato cultivars in the presence or absence of the nematode, *Meloidogyne incognita* (Kofoid and White, 1912; Chitwood, 1949). The current study was carried out to complement and document promising effectiveness of local biocontrol agents against *M. incognita* in another study (Abd-Elgawad and Kabeil, 2010).

Since the pathogen-induced production of pathogenesis-related (PR) proteins of plants is a widespread phenomenon that is being intensively investigated with respect to the underlying signaling pathways as well as to its potential use as markers of plant resistance to nematodes (Abd-Elgawad and Molinari, 2008; Heil and Bostock, 2002), we applied *T. harzianum* and *S. marcescens* as non-pathogenic inducers of resistance to test their efficacy on reducing development and reproduction of the nematode. We additionally investigated enzymatic activities of poly-phenol oxidase (PPO) and β-1,3-glucanases (GLUC) in the tested cultivars to reach more solid and sound conclusions of nematode-host interaction for two cultivars with relatively different sensitivity to the nematode (Abd-Elgawad and Kabeil, 2010). It is well known that PPO causes the oxidation of phenolic compounds to quinones, which are more toxic than the original phenols and the positive correlation between levels of PPO and the resistance of plants to pathogens is frequently observed (Mayer, 2006). GLUCs are capable of catalyzing both degradation of cell walls of pathogenic agents, because β-1,3-glucaneses are essential components of the pathogenic nematode cuticle, and hydrolyses the corresponding substrates, thereby releasing biologically active oligosaccharides (elicitors and suppressors) capable of regulating the immune states of plant tissues (Zinov'eva et al., 2001). Therefore, the two enzymes were tested herein as biochemical/phenotypic markers based on their association with the resistance status or response.

MATERIALS AND METHODS

Greenhouse test

Pure cultures of three Egyptian *T. harzianum* isolates were obtainned from the Center of Fungi, Assiut University, Egypt and maintained on potato dextrose agar in Petri plates at 27 ± 5°C.

These local isolates that proved to be promising in a preliminary study were designated as f_1, f_3 and f_8 and cultured on intact sorghum seeds (Haseeb et al., 2005). The final concentration per plate was set at 10^8 CFU/g of the seeds. 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 11 x 19-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 182 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 ratio; 2.5 kg soil/pot) in a greenhouse at 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 active second-stage juveniles (J_2) of *M. incognita*/plant poured in three holes around the plant stem. Ten days after transplanting and immediately after *M. incognita* inoculation, tomato plants were treated as follows: i) 4 ml/pot of Nemaless (a commercial suspension of a safe and local *S. marcescens* isolate having 1 x 10^9 bacterium cells/ml water); Nemaless was obtained from The Agriculture Research Centre, Giza, Egypt; ii) carbofuran at 1 mg ai/kg soil; iii) three fungal isolates of *T. harzianum* separately applied at 50 ml/kg soil. Control treatments were obtained with both untreated and uninoculated plants. Fifty days after nematode inoculation, plants were harvested and roots rinsed with running tap water. Plant fitness (PF) was recorded using the following growth parameters: length of shoots (SL), weight of the upper part of the plants (GW), number of the branches (BN), length of roots (RL) and weight of roots (RW). An index of PF was calculated for each plant, according to the equation:

$$PF = (SL \times GW) + k(RL \times RW) + BN$$

Where, k represents a constant used to normalize the contribution of root growth to that of the upper parts of plant (Molinari and Abd-Elgawad, 2007). To count egg masses, each root system was weighed and three root samples of 1 g each were examined under a stereoscope (x10 magnification) and the number of egg masses was expressed per root system. To estimate the number of eggs, the roots were chopped up and eggs extracted after shaking chopped roots vigorously in 10% bleach solution for 4 min and counted as previously described (Molinari, 2008). The numbers of eggs were expressed per root system.

Enzyme assays

Enzymatic activities of polyphenol oxidase and β-1,3-glucanase were measured 20 days after nematode inoculation. Plants were harvested and roots were thoroughly rinsed with sterilized distilled (SD) water, excised from the shoots, separately dried from excessive water, and weighed. Then, roots were cut with scissors, placed in an ice-cold 0.05 M phosphate buffer, pH 6.5, and ground by hand with a pestle in a porcelain mortar. This coarse homogenate was centrifuged under cooling conditions for 10 min at 10,000 xg. The supernatants were recovered and the precipitates were re-extracted with the same buffer and re-centrifuged. The supernatants were pooled and designated as crude extracts. Polyphenol oxidase activity was determined spectrophotometrically by measuring the increase in absorbance at 475 nm due to the oxidation of 3,4-dihydroxyphenyl alanine; the specific activity was expressed as µmol of the hallachrome production per minute per mg protein (Leonard, 1971). Three replicates representing three tomato plants per treatment were used for each enzymatic assay. The method described by Bradford (1976) was adopted to determine protein content. The method of Abeles et al. (1970) was used to determine β-1,3-glucanase activity. Laminarin (Sigma) was used as substrate and dinitrosalicylic acid as reagent added to equal amount (1:1) of the crude extract to measure reducing sugars. The amount of glucose released was determined by measurements of absorbance

Table 1. Effect of three isolates of *T. harzianum* (f1, f3 and f8), *S. marcescens* and carbofuran on number of egg masses (EM) and eggs of *M. incognita* on tomato cvs Alisa and Super Strain B and index of plant fitness (PF)*.

Treatment	Tomato cv. Alisa			Tomato cv. Super Strain B		
	PF	EM	Egg	PF	EM	Egg
Nematode (N) only	5378±956^{c+}	75±29^{a}	15375±4022^{a}	3253±785^{d}	94±24^{a}	26132±9854^{a}
T. harzianum f_1 + N	10674±1978^{b}	20±8^{b}	2046±896^{b}	7648±4279^{b}	26±12^{b}	3488±1435^{b}
T. harzianum f_3 + N	11179±1713^{b}	22±10^{b}	2974±763^{b}	5257±2166^{c}	24±11^{b}	3790±1298^{b}
T. harzianum f_8 + N	9842±1032^{b}	23±9^{b}	3219±1095^{b}	5265±1045^{c}	24±9^{b}	3938±864^{b}
Carbofuran + N	10407±2089^{b}	12±3^{c}	724±332^{c}	9147±1689^{b}	16±5^{c}	1569±484^{c}
S. marcescens + N	12289±5438^{b}	18±7^{bc}	2128±983^{b}	7310±2864^{b}	24±10^{b}	3092±912^{b}
Untreated control	19020±5657^{a}	0^{d}	0^{d}	11255±2473^{a}	0^{d}	0^{d}

*Plants were harvested and analyzed 50 days after inoculation. Values are averages ± SD (n = 14). $^{+}$Averages in each column sharing a common letter are not significantly (P ≤ 0.05) different according to Duncan's new multiple range test.

at 530 nm after 1 h. β-1,3-Glucanase activity was expressed as concentration in mM of glucose equivalent released from 1 g fresh weight tissue in 60 min. The pots were arranged in a randomized complete block design with 7 replicates (pots) for nematode-tomato interaction test in addition to the set of plants to do the enzyme analysis and the whole experiment was repeated once. Data were pooled together for statistical computation using ANOVA and Duncan's new multiple range test for mean separation if differences were not statistically (P ≤ 0.05) significant between the cultivars. Egg numbers were transformed to log (x+10) before statistical analysis to achieve normal distribution; actual numbers were presented. Student's t-test in groups (cultivars) was applied for comparison between, both the PF and the enzymatic activity of the two cultivars for untreated as well as uninoculated plants.

RESULTS

According to plant fitness index (PF), tomato plants belonging to cv. Super Strain B had a reduced (P ≤ 0.05) growth with respect to cv. Alisa plants for both untreated and uninoculated plants. Super Strain B was a better host for *M. incognita* (Table 1). *M. incognita* infestation adversely affected (P ≤ 0.05) the plant growth parameters of the two cultivars in all treatments when compared with uninoculated controls. Generally, infested treated plants showed higher PF values than infested untreated controls; PF values of plants treated with carbofuran were the highest, decreasing PF values were obtained by treatments with *S. marcescens*, *T. harzianum* f_1, *T. harzianum* f_3 and *T. harzianum* f_8, respectively. Both cultivars were highly susceptible to nematode attack according to nematode galls on roots as counted and rated on a 0 - 5 scale, known as gall index (Taylor and Sasser, 1978). No galls were found on roots of the control plants, demonstrating lack of contamination. The nematode final population measured by number of nematode eggs increased above the initial population in all treatments except in cv. Alisa plants treated with carbofuran. The nematode-reproduction factor ranged from 0.7 and 1.6 in carbofuran treatment to 15.4 and 26.1 in untreated inoculated cvs. Alisa and Super Strain B, respectively. On the other hand, the nematode final population measured by number of eggs in untreated plants had up to 21 and 17 fold increase over that of treated cvs. Alisa and Super Strain B, res-pectively. All treatments caused a significant and marked decrease of egg masses and eggs per root system when compared with untreated plants. This decrease generally correlated with an acceleration of plant fitness (Table 1). Moreover, treatments with both biocontrol agents and carbofuran lowered female fertility (eggs/egg masses ratios) of nematodes. Apparently, treatment with carbo-furan was the most effective in reducing nematode infestation factors, although plants of cv. Alisa treated with *S. marcescens* showed number of egg masses/root system which was not significantly different (P ≤ 0.05) from those of carbofuran-treated plants.

T-test revealed no significant differences (P ≤ 0.05) in the activities of β-1,3-glucanase or polyphenol oxidase between the two tomato cultivars for untreated and uninoculated plants. Thus, root activity of both β-1,3-glucanase and polyphenol oxidase were summed together and recorded in treated and untreated susceptible tomato inoculated with *M. incognita* (Table 2). Also, no significant changes in β-1,3-glucanase activity were found in treated and untreated inoculated roots with respect to untreated controls. On the contrary, inoculated roots treated or not treated with the isolate *T. harzianum* f_8 showed an increased PPO activity when compared with uninoculated untreated control; treatments with the other biocontrol agents and carbofuran did not induce a comparable increase in PPO activity upon nematode inoculation.

DISCUSSION

Instead of using several separate plant growth parameters, the present study combined them in an index of plant fitness according to Molinari and Abd-Elgawad (2007). This index (Table 1) which added the number of plant branches to the common previous parameters

Table 2. β-1,3-Glucanases (GLUC) and polyphenol oxidase (PPO) activities in roots of susceptible tomato grown in soil treated with three different isolates of *T. harzianum* (f1, f3 and f8), *S. marcescens* or carbofuran and/or *M. incognita* as compared to untreated plants*.

Treatment	GLUC[+]	PPO[++]
Nematode (N) only	67.54±8.33[n.s.]	0.098±0.057[a]
T. harzianum f_1+N	64.63±11.74	0.068±0.032[b]
T. harzianum f_3 +N	63.74±7.19	0.063±0.016[b]
T. harzianum f_8+N	56.01±13.45	0.098±0.068[a]
Carbofuran + N	60.29±13.86	0.045±0.013[b]
S. marcescens +N	68.52±17.35	0.063±0.017[b]
Untreated control	62.25±15.26	0.062±0.013[b]

*Values are averages ± SD (n = 12). Averages of the two cultivars, Super Strain B and Alisa were summed together because differences were not statistically (P ≤ 0.05) significant; n.s.= not significant. [+]β--1,3Glucanase activity was expressed as concentration (units/ml) in mM of glucose equivalent released from 1 g fresh weight tissue in 60 min. [++]The specific activity was expressed in µmol of the reaction product per minute per mg protein. Averages in each column sharing a common letter are not significantly (P ≤ 0.05) different according to Duncan's new multiple range test.

proved to be a sound and comprehensive indicator. The present study confirmed the preliminary results .Although chemical nematicides like carbofuran, may demonstrate high effectiveness against the root-knot nematodes, the induction of resistance by microorganisms such as *Serratia marcescens* and *Trichoderma harzianum* may be considered a more natural and environmentally acceptable alternative to such chemicals. The marked decrease of egg masses and eggs per root system caused by the applied microorganisms (Table 1) is especially important because an estimate of nematode reproduction based on the number of eggs/eggmass, but not galling index, is necessary to determine the nematode fecundity on plant cultivars. Thus, the overall goal of such biological control 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. Commercial products of *T. harzianum* are available (Harman, 2000) but local ones may be more adapted and less expensive without significant risk to Egyptian fauna and flora. The relatively high efficacy demonstrated by *T. harzianum* f_1 (Table 1) supports the need 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 plant fitness and in suppression of root-knot nematode reproduction, chemicals can be replaced to some extent by microbial antagonists viz. *S. marcescens* and *T. harzianum* isolates to reduce the impact on the environment. Moreover, 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.

In tomato roots infected with root-knot nematodes, genes with homology to several known plant defense genes such as 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). This latter study proved that *T. harzianum* elicited induced systemic resistance (ISR) mechanisms in cucumber plants. So, elicited by a local infection of *T. harzianum* and *S. marcescens*, both cultivars of tomato plants may possibly respond with a Salicylic-dependent signalling cascade that leads to the systemic expression of a broad spectrum and long-lasting disease resistance that is efficient against the invading pathogen (Heil and Bostock, 2002). Such a response may include changes in cell wall composition, *de novo* production of pathogenesis-related proteins such as chitinases and glucanases, and synthesis of phytoalexins which are associated with resistance, and further defensive compounds are likely to exist but remain to be identified (Heil and Bostock, 2002).

In this respect, experimental data did not confirm the involvement of β-1,3-glucanase in the mechanism of resistance induction of the ISR elicitors tested in roots of the tomato cvs Super Strain B and Alisa (Table 2). Hence, there is also a possibility of parasitism (Harman, 2000) since the microorganisms were applied to the roots (soil) where the nematode was inoculated. Yet, such a non-ISR possibility is more favored in the applied carbofuran which is well known to have a direct effect on killing a large portion of the nematodes, resulting in fewer nematodes in the plant. On the contrary, reduction of nematode reproduction by possibly ISR elicitors was associated with unchanged polyphenol oxidase activity, except inoculated roots treated with the isolate *T. harzianum* f_8, with respect to uninoculated plants, while full nematode development in untreated plants occurred in relation to a signi-

ficant increase of this enzyme activity. Defense gene transcription or enzyme activity is, most of the time, delayed and lower in compatible (susceptible plant) than in incompatible (resistant plant) interactions (Williamson and Hussey, 1996). Hence, activity of defense-related enzymes was measured 20 days after nematode inoculation (Table 2). Admittedly, the measurements should have been done few days after the inoculation, especially if nematode-resistant tomato cultivar(s) had been included. Plant defense reaction is usually observed in early infection stages (Molinari and Abd-Elgawad, 2007; Williamson and Hussey, 1996). In compatible interaction, the chitinase and glucanase activities in cucumber plants were measured 21 days after *M. incognita* infestation (Zinov'eva et al., 2001).

On the other hand, Desender et al. (2007) suggested that plant-pathogen reaction patterns are, as a rule, specific to each plant genotype/elicitor pair, irrespective of the compatibility/incompatibility status of the interaction. Therefore, if the defense reactions do not depend primarily on genes related to the type of interaction induced locally in tomato roots infected with root-knot nematodes, the specificity of tomato-root-knot nematode interactions concerning the type of elicitor and the plant genotype should be targeted as well. The present study characterized such patterns of two genotype/elicitor pairs (Tables 1 to 2). The fact that β-1,3-glucanase was not found to be involved in the mechanism of resistance induction by the ISR elicitors tested on susceptible tomato does not negate the probability that other pathogenesis-related (PR) proteins generation may contribute to plant protection against parasitic nematodes in the studied interactions (Mohamed and Abd-Elgawad, 2003; Molinari and Abd-Elgawad, 2007).

On the other hand, some authors reported the absence of induction of glucanase in plants infested with nematodes (Oka et al., 1997), while others showed that nematode infestation induces an increase in the activity of this enzyme (Zinov'eva et al., 2001). Yet, because the tomato cultivars studied herein are susceptible to *M. incognita*, the infestation-induced activation of polyphenol oxidase can hardly be regarded as a protective reaction. More probably, activation of some enzymes should be attributed to the substrate induction caused by plant-tissue degradation (Zinov'eva et al., 2001). Another possibility is their limited contribution to the immune response of the plant tissue. Zinov'eva et al. (2001) reported that the enzyme function had a dualistic component which is often mutually exclusive. Although, these enzymes catalyze degradation of the pathogen's cell wall and formation of protective elicitors, thus increasing plant resistance, they may catalyze degradation of immunosuppressor substrates inducing plant susceptibility.

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. Finally, data presented in this paper shows that *T. harzianum* and *S. marcescens* may be used to prime tomato plants for root-knot nematode resistance, although further research should be carried out to under-stand the mechanisms by which this induction occurs

ACKNOWLEDGEMENT

This work was carried out within the bilateral mobility grant "Technology transfer and innovation: Innovative technology for screening resistance against plant-parasitic nematodes of Solanaceae", based on an agreement for scientific and technological cooperation between the Foreign Ministry of Italy and the Egyptian Ministry of Scientific Research. Sincere thanks to Ahmed Elhahrawy and Dr. Sergio Molinari, from Plant Protection Institute, CNR, Bari, Italy, for technical assistance and revising an earlier version of the manuscript; respectively.

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