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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Biological control against the root-knot nematode, *Meloidogyne incognita* was proven to occur in tomato, *Solanum lycopersicum*, 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 × 10⁸ bacterium cells/ml water) 2 ml suspension/kg soil; three different *T. harzianum* isolates (f₁, f₃ and f₉) were separately added at 50 × 10⁸ CFU/kg soil. The possible ISR elicitors were tested on two tomato cultivars (Super Strain B and Alisa), which were inoculated with active juveniles (J₂) 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 ≤ 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...
and Osman, 1977; Greco et al., 1992). Nematode resis-
tant tomato, especially the hybrid cultivars as a non-
chemical strategy may be used under protected cultiva-
tion 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 biol-
ogical 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 com-
mon 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 pathogene-
sis-related (PR) proteins of plants is a widespread phe
nomenon 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 nema
todes (Abd-Elgawad and Molinari, 2008; Heil and
Bostock, 2002), we applied T. harzianum and S. marces-
cens 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-gluc-
canases (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 capa-
bile of catalyzing both degradation of cell walls of patho-
genic agents, because β-1,3-glucanases are essential
components of the pathogenic nematode cuticle, and
hydrolyses the corresponding substrates, thereby relea-
sing 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 obtain-

ed from the Center of Fungi, Assiut University, Egypt and main-
tained on potato dextrose agar in Petri plates at 27 ± 5°C.
The supernatants were recovered and the precipitates were re-extracted
with the same buffer and re-centrifuged. The supernatants were
cultured on intact sor-

ghum seeds (Haseeb et al., 2005). The final concentration per plate
was set at 10⁶ CFU/g of the seeds. Seeds of two tomato cultivars,
Super Strain B and Alisa, were germinated in small (3.5-cm diami-
ter) foam wells filled with sterilized peat and a single seedling
per well of 11 × 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 88%, 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 Hoa-
gland's nutrient solution. Nematode-treated pots were inoculated
with a suspension containing 1000 ± 5 active second-stage juve
nies (J2) 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.
mariescens isolate having 1 x 10⁶ bacterium cells/ml water); Nema-
less was re-suspended from The Agriculture Research Cen
inoculation, tomato plants were treated as follows: i) 4
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ml/pot of Nemaless (a commercial suspension of a safe and local S.
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 f₁*, *T. harzianum f₃* and *T. harzianum f₈*, 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 Sassar, 1978). No galls were found on roots of the control plants, demonstrating lack of contamination. 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, respectively. 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 f₈* 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...
Glucanase activity was expressed as concentration (units/ml) in mM of glucose equivalent released.

Harman, Sharon et al., 2001

Activity was expressed in µmol of the reaction product per minute per mg protein.

Heil and Bostock, 2002

Galacturonase activity of the reaction product per minute per mg protein.

Parvatha Reddy et al., 1996

Glucanase in the mechanism of 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 f8, with respect to uninoculated plants, while full nematode development in untreated plants occurred in relation to a signi-

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*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GLUC*</th>
<th>PPO**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nematode (N) only</td>
<td>67.54±8.33±s.a</td>
<td>0.098±0.057±a</td>
</tr>
<tr>
<td>T. harzianum f1+N</td>
<td>64.63±11.74±b</td>
<td>0.068±0.032±b</td>
</tr>
<tr>
<td>T. harzianum f3+N</td>
<td>63.74±7.19±b</td>
<td>0.063±0.016±b</td>
</tr>
<tr>
<td>T. harzianum f8+N</td>
<td>56.01±13.45±b</td>
<td>0.098±0.068±b</td>
</tr>
<tr>
<td>Carbofuran + N</td>
<td>60.29±13.86±b</td>
<td>0.045±0.013±b</td>
</tr>
<tr>
<td>S. marcescens +N</td>
<td>68.52±17.35±b</td>
<td>0.063±0.017±b</td>
</tr>
<tr>
<td>Untreated control</td>
<td>62.25±15.26±b</td>
<td>0.062±0.013±b</td>
</tr>
</tbody>
</table>

*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. **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.
significant 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 understand the mechanisms by which this induction occurs.

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