Use of *Trichoderma harzianum* and *Trichoderma viride* for the Biological Control of *Meloidogyne incognita* on Tomato

*A. Dababat* and *R. Sikora*

**ABSTRACT**

*Trichoderma harzianum* and *Trichoderma viride* were tested for their capacity to reduce the incidence and pathogenicity of the root-knot nematode *Meloidogyne incognita* on tomato. *In vitro* studies demonstrated that all tested isolates were effective in causing second-stage juvenile (J2) mortality compared with the control. However, a slight increase in J2 mortality coincided with the use of *Trichoderma harzianum* (Th3) when compared to the other *Trichoderma* isolates. *Trichoderma* slightly reduced nematode damage to tomato *in vivo*. Treatment of the soil with the biocontrol agents slightly improved nematode control when applied one week before transplanting, but not at transplanting time. The *Trichoderma* isolates could not be re-isolated from the endorhiza, but were successfully re-isolated from the rhizosphere 45 days after fungal inoculation. Only slight increases in plant growth could be measured. The mutualistic endophyte *F. oxysporum*162, used as positive control, was significantly more effective in root-knot nematode biocontrol than the *Trichoderma* isolates.

**Keywords:** *Meloidogyne incognita*, Tomato, *Trichoderma harzianum*, *Trichoderma viride*.

**1. INTRODUCTION**

Biological control of soil-borne plant pathogens and plant parasitic nematodes by antagonistic microorganisms is a potential non-chemical means of plant disease control (Papavizas, 1985; Kerry, 1987; Stirling, 1991). A wide range of bacteria (Hallmann et al., 2001) and fungal agents (Spiegel and Chet, 1998; Kerry, 2000; Meyer et al., 2001) have been used to reduce a range of plant parasitic nematodes.

Some species of *Trichoderma* have been used widely as biocontrol agents against soil-borne plant diseases (Harman, 1991; Tronsmo, 1996; Rabeendran et al., 1998; Whipps, 2001). *Trichoderma* isolates have been used successfully to control the damage caused by soil-borne pathogens in greenhouses and under opened-field conditions (Papavizas, 1985). *Trichoderma* also have been shown to have activity towards root-knot nematode (Windham et al., 1989; Sharon et al., 2001). Some *Trichoderma* isolates were reported to do both enhance plant growth and reduce root-knot nematode damage (Windham et al., 1989; Meyer et al., 2001).

Fungal pathogens are killed by the releases of toxic compounds (e.g. the antibiotics gliotoxin, gliovirin, and peptabiols) and a battery of lytic enzymes, mainly chitinases, glucanases, and proteases produced by species of *Trichoderma*. These enzymes facilitate penetration into the host and the utilization of host nutrients (Lorito et al., 1996).

Direct pathogenicity of fungal biocontrol agents is one of the main mechanisms responsible for plant parasitic nematode control (Kerry, 1987; Stirling, 1991). However, secondary metabolites from fungi also contain compounds which are toxic to plant parasitic nematodes.
Use of *Trichoderma harzianum* … Abd Al-Fattah A. Dababat and Richard A. Sikora

(Hallmann and Sikora, 1996; Sikora et al., 2003). Culture filtrate of different biocontrol agents, effective against plant parasitic nematodes, was previously studied by many researchers (Reißinger, 1995; Hallmann and Sikora, 1996) and more recently by Meyer et al. (2004) and Vu (2005).

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 (Spiegel and Chet, 1998; Dababat et al., 2006).

A number of *Trichoderma* isolates are now used commercially for the control of fungal pathogens in the soil. These isolates, however, have not been tested for root-knot nematode control. The aims of the following experiments were to: determine the effect of *Trichoderma* as a biocontrol agent for controlling the root-knot nematode *M. incognita*, test the effect of time of inoculation on control efficacy, study the effect of the different isolates on plant growth promotion, investigate direct parasitism of *Trichoderma* towards *M. incognita*, and examine the influence of secondary metabolites on J2 of *M. incognita* mobility and mortality.

2. MATERIALS AND METHODS

*Meloidogyne incognita* was multiplied on tomato plants cv. Furore. 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 *Trichoderma* isolates were cultured on Potato Dextrose Agar (PDA) containing 150 mg l⁻¹ streptomycin and 150 mg l⁻¹ of chloramphenicol. The Petri dishes were then placed in an incubator at 24°C for 1-2 weeks. The mycelia and conidia formed were then carefully scraped from the media and suspended in 100ml tap water. Spores were separated from mycelia by sieving through a 50µm sieve. The spore suspensions were then adjusted to the desired concentration after counting spore density using a Thoma haemocytometer.

Tomato cv. Hellfrucht Frühsämm which is highly susceptible to *M. incognita* was used in this study.

The endophytic non-pathogenic *F. oxysporum* 162, isolated from tomato endorhiza tissue, was used in this study as a positive control because of its high antagonistic potential (Hallmann and Sikora, 1994).

Experimental Procedures

I. Influence of Duration of Exposure of *Trichoderma* to *Meloidogyne incognita* on Biocontrol

Soil Treatment at Planting

Tomato seeds of cultivar Hellfrucht Frühsämm were sown in a commercial style 70 plug seedling trays, with each plug measuring 4 x 4.5 x 2.5 cm. Seedlings were then propagated for 3 weeks in the greenhouse, before being transplanted into new pots (7x7x8 cm) containing 400g of a mixture of sterilized sand: soil (2:1, v/v), respectively. One week after transplantation, the seedlings were inoculated with one of the following fungi: *T. viride* (Tv), *T. harzianum* (Th1), *T. harzianum* (Th2) or the positive control *F. oxysporum* (FO162). Each seedling received 1 ml of a liquid suspension of the *Trichoderma* isolates containing 5 x 10⁶ spores ml⁻¹ or 1.5 x 10⁷ spores ml⁻¹ of FO162. 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 tap water. The pots were then immediately inoculated with a 3-ml tap water suspension containing 650 J2 of *M. incognita*. The inoculum was also injected into 3 holes roughly 2-cm deep around the stem base.

The experiment consisted of six treatments: 1) Tv + Mi, 2) Th1 + Mi, 3) Th2 + Mi, 4) FO162 + Mi, 5) Mi, and 6) absolute control. The treated pots were incubated in the greenhouse at 22°C ±5 with 16 hours of supplemental artificial light per day. The plants were watered and fertilized with 2 g per liter water (N:P:K =14:10:14) to ensure proper plant growth. The plants were periodically examined for phytotoxicity.

The experiment was terminated 8 weeks after fungal
and nematode inoculation. Fresh shoot weight as well as plant height from the base of the stem to the growing point was measured. The roots were removed, washed free of soil and stained in 0.015% Phloxine B for 20 minutes to facilitate egg mass counting (Shurtleff and Averre III, 2000). The number of galls and egg masses per plant was then determined.

*Trichoderma* re-isolation from the soil was evaluated by taking soil samples from 5 replicates. Samples were taken from 3-cm deep using cork borer. A total of 15 g soil of each treatment was collected. *Trichoderma* were isolated from the soil by dilution plating technique on 9-cm diameter Petri dishes, using PDA medium. The Petri dishes were incubated at 25°C in the dark. One week after plating, pure culture was done to check for the presence of inoculated isolates. Furthermore, 12 root sections were taken from all plants inoculated with FO162 and from the absolute control, and used for re-isolation of FO162 to determine the level of endophytic colonization. Endophytic colonization was determined after sterilization of the root surface with 1.5% NaOCl for three minutes, followed by three rinses in sterilized water. Root sections of approximately 0.5-cm length and of a uniform size of approximately. 0.5-1 mm diameter were removed and placed onto PDA Petri plates containing 150 mg l⁻¹ streptomycin and 150 mg l⁻¹ chloramphenicol (Hallmann and Sikora, 1994). Successful re-isolation was confirmed when the growth characteristics of the fungus and its colour on PDA, as well as the shape of the conidia corresponded with that of the original isolate.

**Soil Treatment before Transplanting**

The same general procedures mentioned above were followed in this test. However, the soil in the pots was treated with the biocontrol agents in addition to 5000 nematode eggs one week before transplanting the tomato seedlings. In this experiment, *T. harzianum* (Th3) was also tested in addition to the other fungal isolates used in the first experiment.

This test was designed to determine direct pathogenicity of the different *Trichoderma* isolates against *M. incognita* J2 in soil in the absence of tomato. A one-ml spore suspension of *Trichoderma* at 5x 10⁶ spores or FO162 containing 1.5x 10⁷ spores was inoculated into 100 cm³ pots filled with 100 g sterile sand obtained by sieving through 500-µm aperture sieve. Fungal and nematode inoculation was done as in experiment 1.1. Tap water was used as a control. Pots were placed in the greenhouse at 24°C, and watered daily. One day after fungal treatment, a one ml solution containing 1000 J2 of *M. incognita* was added to each pot in three 2-cm deep holes. The level of mortality was determined after one week by extracting the J2 from the sand by adding the sand to a 2 liter bottle filled with tap water. The bottle was agitated and the supernatant then passed through a 20-µm sieve. The nematodes collected on the 20-µm sieve were examined and active and inactive nematodes counted. The experiment was repeated a second time.

**III. In vitro Effects of Metabolites of Trichoderma on Mobility and Mortality of *M. incognita***

*Trichoderma* isolates were first cultured on PDA in an incubator at 25°C for one week. Five 0.5-cm plugs of PDA with fungal mycelia were added to flasks containing 200 ml of Potato Dextrose Broth (PDB). The flasks were shaken in an incubator at 25°C for one week. Fungal mycelia were removed from the broth by filtration through 3 layers of cheese cloth. The suspension was then centrifuged at 5000 rpm for 20 min at 20°C to remove small sections and spores. The supernatant was filtered through a combination of 0.45 µm and 0.20 µm pore size micro-filters. Approximately, 0.3 g of streptomycin sulphate was added to the solution so that the final antibiotic concentration was 150 ppm to prevent microbial growth. Sterile water as well as filtrate from 5 un-inoculated PDA pieces in PDB medium was used as the absolute control. The experiment was conducted under sterile conditions and set up in 6 multi well-trays. For each treatment, 3 ml of sterile metabolite solution was combined with 3 ml sterile water containing 1000
sterile J2 in each well for a final concentration of 50%. A 90% solution was prepared by combining 5.4 ml of metabolite solution with 0.6 ml of sterile water containing 1000 sterile J2 of *M. incognita*. The multi well-trays were stored in an incubator at 25°C and mobility of the J2 was measured after 24 hours at a concentration of 90% and after 4 days at the 50% concentration. A one-ml suspension of J2 from each well was transferred to a counting chamber and mobility of 100 nematodes was recorded. Nematodes which were inactivated were added to tap water for 24 hours and those which were still immobile and straight were considered as dead (Hallmann and Sikora, 1996), and percent of mortality was calculated.

3. RESULTS

I. Influence of Duration of Exposure of *Trichoderma* to *Meloidogyne incognita* on Biocontrol

Soil Treatment at Transplanting

Simultaneous treatment of the soil at transplanting with *Trichoderma* and *M. incognita* resulted in a reduction of up to 19.5% in nematode galling. A significant reduction in gall formation was obtained with *Trichoderma harzianum* (Th1) when compared to the control. However, in both experiments, the FO162 standard gave higher levels of control when compared to all *Trichoderma* isolates (Figure 1). The suppressive effect of the fungi on egg mass production, which is a measure of nematode development over time, was confirmed only for FO162. *Trichoderma* isolates did not reduce egg mass number (Figure 1).

Treating the soil simultaneously at transplanting with *Trichoderma* or *F. oxysporum* 162 and *M. incognita* had no significant effect on shoot height (Figure 2 a). Shoot weight, however, slightly increased when the plants were inoculated with *Trichoderma* isolates Th1 and Th2 or FO162 compared to the absolute control (Figure 2 b). The *Trichoderma* isolates could not be re-isolated from the endorhiza, but were successfully re-isolated from the rhizosphere 45 days after fungal inoculation (data not shown).

Soil Treatment before Transplanting

Inoculating the soil with *Trichoderma* and *M. incognita* one week before transplanting tomato seedlings resulted in reductions of up to 30.8% in nematode galling. The differences, however, were not significant due to high variation. The FO162 isolate, on the other hand, caused a significant reduction of 44.5% in nematode galling (Figure 3).

*Trichoderma viride* caused a significant reduction in egg mass number by 20%. Whereas, FO162 reduced egg mass formation 50% over the absolute nematode control (figure 3). In contrast to the treatment at planting, where no effect on egg mass number was observed, most of the *Trichoderma* isolates were able to induce a slight reduction in egg mass number (up to 9%) when added to the soil one week before transplanting (Figure 3).

The results of pre-inoculating soil with *Trichoderma* and the nematode before planting had no significant effect on shoot height (Figure 4 a), Th1 and FO162 caused a slight increase in height. Shoot weight increased slightly but not significantly when the plants were inoculated with the *Trichoderma viride* and *Trichoderma harzianum* (Th2) as well as with FO162 when compared to both the absolute and nematode controls (Figure 4 b).

II. Pathogenicity of *Trichoderma* toward *M. incognita* in Soil in the Absence of Tomato

Nematodes J2 mortality as affected by the three *Trichoderma* isolates ranged from 36-40% and was significantly higher when compared to the nematode controls (Figure 5). There were no significant differences in the activity between the *Trichoderma* isolates. FO162 when compared with the *Trichoderma* isolates gave superior nematode control of up to 80%.

No growth of *Trichoderma* or FO162 on the nematode cuticle or parasitic inside the nematode was detected. Only in one case *Trichoderma harzianum* (Th3) was observed parasitizing the J2.

III. *In vitro* Effects of Metabolites of *Trichoderma* on Mobility and Mortality of *M. incognita*

Exposing the nematode to culture filtrates of
Trichoderma at a concentration of 90% significantly decreased mobility of M. incognita J2 after 24 hours when compared to the water control. Mortality after testing recovery of inactive nematodes in tap water was still high and ranged between 14.3- 38.3% in the treatments compared to 3% and 9.7% in the water and media controls, respectively (Figure 6).

Significant differences between the different biocontrol agents were found. The highest level of nematode mortality was obtained by Trichoderma harzianum 3, which produced nematode mortality of 38% compared with 14.3%, 27% and 32.7% for Tv, Th1 and Th2 isolates, respectively.

Culture filtrates of all fungi at a concentration of 50% decreased nematode mobility significantly after 4 days over the water control. Nematode immobility ranged from 10 to 28% among the biocontrol agents compared with 7.8 % and 1.3% in the media and water controls, respectively (Figure 7). FO162 gave the highest level of inactivation after 4 days. All Trichoderma harzianum isolates had a significant effect on nematode mobility at concentration of 50% and after 4 days; except for Trichoderma viride, which gave the lowest value of immobility (10%) and was significant only when compared with absolute control (water) but not with the media control. However, the level of control of the Trichoderma isolates was far below that of the FO162 standard which gave nematode immobility of about 28%.

4. DISCUSSION

I. Influence of Duration of Exposure of Trichoderma to M. incognita on Biocontrol

Treatment of the soil with the Trichoderma isolates at the time of transplanting resulted in a small reduction in nematode galling. However, galling was significantly reduced when treatment took place one week before transplanting - allowing for extended exposure of the nematode to the antagonist. In both simultaneous inoculations one week before or at transplanting, treatment of the plants with the standard biocontrol agent FO162 reduced the number of egg masses, while Trichoderma isolates gave only slightly reductions.

Similar results were obtained by other researchers with Trichoderma. Sharon et al. (2001) reported that Trichoderma harzianum reduced galling of root-knot nematode Meloidogyne 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 Trichoderma harzianum preparations in short term trials. Similar results were obtained by Pandey et al. (2003) using different treatments of Trichoderma viride against M. incognita in chickpea. All Trichoderma viride treatments decreased galling and the final nematode population densities of M. incognita in both field and pots experiments as the level of the T. viride increased. Number of eggs and second-stage juveniles (J2) per gram root were significantly lower with fungal treatment.

In the current study, inoculating the seedlings with Trichoderma did not have a consistent positive effect on plant growth. Neither shoot weight nor plant height was clearly affected. The results are similar to 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. Similar results also were obtained by Meyer et al. (2001) with Trichoderma. Conversely, Windham et al. (1986), Kleifeld and Chet (1992), Spiegel and Chet (1998), Sharon et al. (2001), and Pandey et al. (2003) reported a stimulatory effect of Trichoderma spp. on plant growth.

All Trichoderma isolates could be re-isolated from rhizosphere soil. Re-isolation of the fungi from endorhiza was confirmed for FO162 but not for the Trichoderma isolates. The addition of the fungi did not cause disease symptoms to appear on the plants.

The level of control with Trichoderma, obtained in the present tests, using short exposure times to juveniles, did not prove effective for further field development of these isolates as a commercial product. One week pre-exposure or simultaneous treatment at transplanting gave small to moderate levels of control, not sufficient for

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practical application. The level of biocontrol could be improved significantly, if the nematode is exposed to the *Trichoderma* for longer periods of time before transplanting. For example, treatment during the preparation of raise-beds in vegetable production would give the antagonist a 5-week period for parasitism before transplanting takes place. Experimentation is now being conducted to improve the timing of treatment of *Trichoderma* to attain practical levels of biocontrol of root-knot control.

### II. Pathogenicity of *Trichoderma* toward *M. incognita* in Soil in the Absence of Tomato

The results obtained from the interaction between the different biocontrol agents and *M. incognita* in the absence of tomato showed a direct effect of *Trichoderma* and FO162 on *M. incognita*. This was demonstrated in the high mortality levels of the nematodes when the sand was treated with the different biocontrol agents. *Trichoderma* isolates were not as effective as FO162. *Trichoderma* may be effective as an egg pathogen; and a pre-plant treatment when eggs are present, not J2, may give better results.

### III. *In vitro* Effects of Metabolites of *Trichoderma* on Mobility and Mortality of *M. incognita*

The effect of culture filtrates of the *Trichoderma* on *M. incognita* mobility was clearly demonstrated in the present study. Culture filtrates of *Trichoderma* at concentrations of 50% and 90% caused nematode immobility ranging from 14.3-38% and 11-22 %, respectively.

Previous studies carried out by Amin (1994) and Hallmann and Sikora (1996) showed that metabolites of endophytic *F. oxysporum* strains decreased significantly the mobility of migratory endo-parasitic nematodes *in vitro*. Metabolites of *F. oxysporum* isolate B20 from a gliotoxin fermentation medium at a concentration of 50% strongly affected mobility of *Radopholus similis* (Amin, 1994). Similarly, Hallmann and Sikora (1996) showed that the metabolites of *F. oxysporum* strain 162 from a gliotoxin fermentation medium at a concentration of 90% inactivated approximately 65% of J2 and adults of the migratory endoparasitic nematodes *Pratylenchus zeae* and *R. similis*.

In the present study, the metabolites of the *Trichoderma* did not differ from FO162 metabolites in the level of activity. However, they significantly decreased the mobility of *M. incognita* when compared to the nematode control. Mortality was lower after rinsing and storing in tap water for 24 hours. After rinsing and storing in tap water for 24 hours, mortality for *Trichoderma* isolates and FO162 ranged from 14-39% as compared to 3-10% in media and absolute controls, respectively. In previous studies, mortality of root-knot nematode was associated with the disintegration of internal tissue of the nematodes tested caused by culture filtrates of mutualistic endophytic *F. oxysporum* (Amin, 1994; Schuster et al., 1995; Hallmann and Sikora, 1996). Second stage juveniles of the migratory endoparasitic nematode *Radopholus similis* were more sensitive to fungal metabolites than older juvenile stages or adults (Amin, 1994).

**ACKNOWLEDGMENT**

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<th>Isolate code</th>
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<td><em>Fusarium oxysporum 162</em></td>
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Figure 1. Dababat and Sikora.
Influence of simultaneous soil treatment with *Trichoderma harzianum*, *Trichoderma viride*, or *Fusarium oxysporum* 162 and *Meloidogyne incognita* on gall and egg mass number 8 weeks after nematode inoculation at the time of transplanting tomato. Means with (*) are significantly different from the control *Meloidogyne incognita* based on Tukey Test ($P \leq 0.05$; $n = 10$).

Fig. 2 A
Influence of simultaneous soil treatment with *Trichoderma harzianum*, *Trichoderma viride*, or *Fusarium oxysporum* 162 and *Meloidogyne incognita* on shoot height (A) and shoot weight (B) 8 weeks after nematode inoculation at the time of transplanting. NS: no significant difference based on Tukey Test ($P \leq 0.05; n = 10$).

Influence of soil pre-treatment with *Trichoderma harzianum*, *Trichoderma viride*, or *Fusarium oxysporum* 162 and *Meloidogyne incognita* on gall and egg mass number 9 weeks after nematode inoculation of tomato. Means with (*) are significantly different from the control *Meloidogyne incognita* based on Tukey Test ($P \leq 0.05; n = 10$).
Influence of soil pre-treated with *Trichoderma harzianum*, *Trichoderma viride*, or *Fusarium oxysporum* 162 and *Meloidogyne incognita* on shoot height (A) and shoot weight (B) 9 weeks after fungal inoculation. NS: no significant difference based on Tukey Test ($P \leq 0.05$; $n = 10$).

Figure 4 (A and B) Dababat and Sikora.
Use of *Trichoderma harzianum* … Abd Al-Fattah A. Dababat and Richard A. Sikora

**Figure 5.** Dababat and Sikora. Effect of *Trichoderma harzianum*, *Trichoderma viride*, or *Fusarium oxysporum* 162 on *Meloidogyne incognita* mortality in soil in the absence of tomato. Means with different letters in the same column are significantly different based on Tukey Test ($P \leq 0.05$; $n = 8$).

**Figure 6.** Dababat and Sikora. Effect of metabolites of *Trichoderma harzianum*, *Trichoderma viride*, or *Fusarium oxysporum* 162 on *Meloidogyne incognita* mortality after 24 hours *in vitro* (90% concentration). Means with different letters in the same column are significantly different based on Tukey Test ($P \leq 0.05$; $n = 6$).
Effect of metabolites of *Trichoderma harzianum*, *Trichoderma viride*, or *Fusarium oxysporum* 162 on *M. incognita* mobility after 4 days *in vitro* (50% concentration). Means with the same letter are not significantly different based on Tukey Test (*P* ≤ 0.05; *n*=6)

REFERENCES


-Trichoderma viride \( \text{vs} \) Trichoderma harzianum \( \text{vs} \) Trichoderma viride

**ت. أوبز**

1. **بـقاؤ وثائق الكائنات المحبّة على بذور المبيدات السامة**
   - Trichoderma harzianum
   - Trichoderma viride

**مقدمة**

- Trichoderma harzianum
- Trichoderma viride

- "Trichoderma viride" كائنات حية محبّة

**المواد والطريقة**

- نتائج تحميل Trichoderma harzianum
- نتائج تحميل Trichoderma viride

**الناتج**

- Trichoderma harzianum
- Trichoderma viride

**الخلاصة**

- "Trichoderma viride" كائنات حية محبّة

**الكلمات المفتاحية**

- Trichoderma harzianum
- Trichoderma viride

**المراجعات**

- Trichoderma harzianum
- Trichoderma viride