Biological Control of the Root-Knot Nematode *Meloidogyne javanica* by *Trichoderma harzianum*

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ABSTRACT

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The fungal biocontrol agent, *Trichoderma harzianum*, was evaluated for its potential to control the root-knot nematode *Meloidogyne javanica*. In greenhouse experiments, root galling was reduced and top fresh weight increased in nematode-infected tomatoes following soil pretreatment with *Trichoderma* peat-bran preparations. The use of a proteinase Prb1-transformed line (P-2) that contains multiple copies of this gene improved biocontrol activity in the greenhouse experiments compared with the nontransformed wild-type strain (WT). All the *Trichoderma* strains showed the ability to colonize *M. javanica*-separated eggs and second-stage juveniles (J2) in sterile in vitro assays, whereas P-2 also penetrated the egg masses. This protease-transformed line presented the same nematicidal and overall proteolytic activity as the WT in in vitro tests in which concentrated soil extracts from *Trichoderma*-treated soils immobilized the infective J2. However, the J2 immobilization and proteolytic activities of both P-2 and the WT were higher than those obtained with strain T-203. Characterization of the activity of all *Trichoderma* strains soil extracts on J2 showed that it was heat resistant and restricted to the low-molecular-weight fraction (less than 3 kDa). It is suggested that improved proteolytic activity of the antagonist may be important for the biological control of the nematodes.

Additional keywords: plant-parasitic nematodes.

Plant-parasitic nematodes cause great economic losses to agricultural crops worldwide (26). Due to the problems caused by chemical control, development of alternative control measures is of great importance. The root-knot nematodes (*Meloidogyne* spp.) are sedentary endoparasites and are among the most damaging agricultural pests, attacking a wide range of crops (3). The infection starts with root penetration of second-stage juveniles (J2), hatched in soil from eggs stored in egg masses that have been laid by the females on the infected roots (3). Some species of the genus *Trichoderma* have been used as biocontrol agents against soilborne, foliar, and postharvest phytopathogenic fungal pathogens (6,7). These fungi may also promote plant growth (18) and have the ability to colonize root surfaces and the cortex (20,32).

Several attempts have been made to use *Trichoderma* spp. to control plant-parasitic nematodes. Windham et al. (31) reported reduced egg production in the root-knot nematode *Meloidogyne arenaria* following soil treatments with *T. harzianum* (T-12) and *T. koningii* (T-8) preparations. Combining *T. harzianum* with neem cakes reduced the population of the citrus nematode *Tylenchulus semipenetrans* (22). Among several other plant-based formulations of *T. harzianum* that were evaluated for the management of *M. incognita*, castor cake extracts showed the best biocontrol activity (23). Reduction of *M. javanica* infection with several isolates of *T. lignorum* and *T. harzianum* has been reported (30).

Various mechanisms have been suggested for the biocontrol activity of *Trichoderma* spp. against phytopathogenic fungi: antibiosis, competition, mycoparasitism, and enzymatic hydrolysis (11,28). Enzymes such as chitinases, glucanases, and proteases seem to be very important in the mycoparasitic process (17). A mechanism of induced resistance is currently being investigated, and evidence for defense responses induced by *T. harzianum* has been provided (32). All mechanisms, except competition, can potentially be involved in the nematode biocontrol process. Information about the possible mechanisms of this fungal activity against nematodes is very limited; understanding these processes 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 (25). The fungus penetrated the cysts and the eggs in those cysts, resulting in larval death. The effect of *T. viride* metabolites on nematodes was demonstrated by implementing root-dip treatments with the fungal culture filtrate (19).

Proteinases are involved in host–parasite interactions, and correlations between pathogenicity and proteinase activity have been reported for plant pathogens (15). Proteinases are also involved in nematophagous fungal activity on nematode eggs (9), e.g., proteases purified from *Verticillium suchlasporium* (21), *V. chlamydosporium* (27), and *Paecilomyces lilacinus* (5). The involvement of *Trichoderma* proteolytic activity in the biocontrol process of fungal plant pathogens was suggested by Rodriguez-Kabana et al. (24) for *T. viride* and by Elad and Kapat (14) for *T. harzianum* (T-39). A 31-kDa basic proteinase (Prb1), produced by *T. harzianum* IMI 206040, was identified, and the gene encoding this proteinase was cloned and characterized (16). Transgenic lines of *T. harzianum* carrying multiple copies of *prb1* revealed improved biocontrol activity against *Rhizoctonia solani* in cotton plants (15).

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In the present study, we used greenhouse experiments to show the ability of *T. harzianum* isolates to control the root-knot nematode *M. javanica* and the improved activity of a proteinase-transformed strain that contains multiple copies of the *prb1* gene. In vitro mode-of-action studies demonstrated the activity of metabolites derived from *Trichoderma*-treated soil extracts and direct parasitic interactions between the fungus and the nematode.

MATERIALS AND METHODS

Nematodes. The root-knot nematode *M. javanica* was propagated on tomato plants (*Lycopersicon esculentum* Mill cv. 144) in the greenhouse, or aseptically on excised tomato roots in petri dishes for in vitro assays. Eggs were separated from egg masses with sodium hypochlorite (0.5%, 1 min) and hatched in water to produce infective J2.

***Trichoderma* isolates and preparations.** T-203 is an Israeli isolate of *T. harzianum* Rifai (13). P-1, -2, -5, and -6 are transformed lines of *T. harzianum* IMI 206040, a wild-type isolate designated WT. The transformed lines were previously genetically engineered for overexpression of proteinase Prb1 activity (15).

The wheat bran-peat preparations were produced by Mycontrol Ltd., Alon-Hagalil, Israel, according to the method described by Sivan et al. (29). The preparations contained 10^8 CFU/g.

Soil and nematode inoculation. Sandy loam soil (pH 7.8; 0.8% organic matter), taken from a deep layer, free of root debris, was determined to be free of root-knot nematodes and stored dry until use.

For experiments with J2-infested soil, the soil was used as is or mixed with the peat-bran preparations and the J2 were added to the pots 10 days before planting. For experiments with naturally infested soil, *M. javanica*-infected tomato plants were grown in the soil for 2 months and uprooted. This soil, containing egg masses and J2 (≈2,000 J2 per liter), was kept moisturized until use.

Seedlings. Tomato speed-seedlings cv. 144 (Hishtill Nursery, Israel) were used in all the greenhouse experiments, unless otherwise specified. The seedlings were planted with the potting mix.

Activity of *Trichoderma* soil treatments and controls. Activities of the fungal peat-bran preparations, control treatments of the peat-bran carrier without fungus, twice-autoclaved fungal preparation, and nontreated controls were evaluated. Soil was mixed with the T-203 preparation or controls (1%, wt/wt) in 750-ml pots with 10 replicates per treatment, and each pot was inoculated with 2,000 J2. Ten days after *Trichoderma* (T-203) and nematode inoculation of the soil, tomato speed-seedlings were planted. Thirty days after seedling exposure to nematodes, plants were removed and top fresh weights and root-galling indices were evaluated on a scale of 0 to 5 (2). Similar experiments were conducted with naturally infested soil, in which the seedlings were planted 1 month after the soil had been treated with the *Trichoderma* (T-203 or WT) preparations or controls.

TABLE 1. Effect of *Trichoderma harzianum* T-203 amended to *Meloidogyne javanica*-infested soil on tomato seedlings

Treatment[y]	Top fresh weight (gram per plant)[z]	Galling index (1 to 5 scale)[z]
T-203 preparation	23.8 a	0.8 c
Autoclaved peat-bran carrier	22.4 ab	2.4 ab
Autoclaved preparation	20.4 a	2.1 b
Nontreated soil	13.3 c	2.6 a

[y] Experiment was performed in 750-ml pots containing sandy loam soil artificially infested with 2,000 second-stage juveniles and mixed with 10 replicates of *Trichoderma* peat-bran preparation, autoclaved preparation or peat-bran carrier (1%, wt/wt). Ten days later, pots were planted with tomato seedlings.

[z] Top fresh weight and galling indices of the infected roots were determined 1 month after planting. Values within columns followed by different letters are significantly different according to Tukey's test ($P = 0.05$).

Evaluation of the activity of proteinase Prb1-transformed lines. The effect of several proteinase-transformed lines on root-galling and top fresh weight compared with the WT was assessed in J2-artificially infested soil in pot experiments. The soil was mixed with *Trichoderma* preparations (1%, wt/wt) and infested with 2,000 J2 per 750-ml pot with 10 replicates for each treatment. Nontreated nematode-infested soil served as a control. Tomato speed-seedlings were planted 10 days later, and after 1 month, the top fresh weights and galling indices were recorded.

Activity of *Trichoderma* T-203, WT, and P-2 in J2-infested soil. Experiments comparing the effect of T-203, WT, and the chosen proteinase-transformed line, P-2, with a nontreated control, were conducted as previously described, in larger pots of 3 liters infested with 8,000 J2 per pot and 10 replicates for each treatment. Six weeks after planting, the top fresh weights and galling indices were recorded.

Evaluation of *Trichoderma* activity in naturally infested soil. This experiment was performed to test the effect of *Trichoderma* soil treatment in naturally infested soil (rather than artificially J2-infested soil) in larger containers. Fifty-liter containers were half-filled with the naturally infested soil, and in the upper half of the container, the infested soil was mixed (1%, wt/wt) with the fungal preparation (WT). Nontreated nematode-infested soil served as a control. Four weeks after *Trichoderma* WT application, four tomato seedlings were planted in each container. Each treatment included four replicates. Two months after planting, top fresh weights and galling indices were recorded. Fungal survival in the soil was estimated after plant harvest by mixing four 10-g samples of soil from each container of the *Trichoderma* treatment or control and estimating CFU per gram of soil by serial-dilution plating on selective medium (12).

Implementation of *Trichoderma* T-203 to root-balls. The root-ball of the tomato seedlings was supplemented with *Trichoderma* (T-203) preparation during seedling growth in the nursery (Hishtill Nursery), as described by Inbar et al. (18). These seedlings were used to assess the efficacy of the root-ball implementation method in reducing root-knot nematode infection. These experiments were conducted in 3-liter pots containing naturally infested soil, with 10 replicates for the *Trichoderma* root-supplemented seedlings and for the controls (nontreated seedlings). One month after planting, galling indices and top fresh weights were recorded.

Nematode development in roots following *Trichoderma* soil treatment. This experiment was performed to determine whether the fungal soil treatment interferes with nematode penetration to the roots, rather than inhibition of nematode development within the roots. Pots (100 ml) were filled with nontreated soil or with *Trichoderma*-amended (WT, P-2, and T-203) soils (1%, wt/wt), and inoculated with 100 J2 per pot. After 10 days, 3-week-old tomato seedlings (cv. 144) that had been germinated on sandy soil were planted, and 28 days after planting, roots were stained with acid fuchsin (10) to visualize the different nematode life stages inside the roots: second-, third-, and fourth-stage juveniles and adult females.

Greenhouse experimental conditions. All the experiments were conducted in a temperature-controlled greenhouse at 27 ± 3°C. Plants were fertilized with a 20-20-20 nutrient solution (75 mg/liter) and irrigated with plain tap water as needed. After *Trichoderma* strains had been mixed with the soil, the soil was kept wet with minimal irrigation.

Soil extracts. Naturally infested soils from pot experiments treated with *Trichoderma* T-203, WT, and P-2, or controls of autoclaved peat-bran, autoclaved preparations, and nontreated soil were sampled 1 month after application of the treatments. Each sample (100 g of soil) was extracted with 100 ml of distilled water by vigorous shaking for 1 h in a 500-ml Erlenmeyer flask, and allowed to sediment for 30 min. The supernatant was collected,

centrifuged for 10 min at 10,000 × *g*, and the resultant supernatant was filter-sterilized with a 0.22-µm Durapore membrane (Millipore Corp., Bedford, MA). The sterile product was freeze-dried and resuspended in double-distilled water in 10^{-1} of the original volume.

Effect of soil extracts on nematodes. The effect of soil extracts on J2 mobility or hatching from egg masses was tested in vitro in sterile 96 multiwell flat-bottom plates (Corning Costar, Corning, NY). Concentrated soil extract or water (95 µl) was added to each well (five replicates for the J2 bioassay and 10 for the egg mass bioassay). Sterile J2 were suspended in a 5-µl drop of water, and ≈100 J2, or one egg mass (including the gall), were inserted into each well. The plates were incubated at 25 ± 2°C. After 2 days, the percentage of mobile J2 was determined by counting mobile versus nonmobile (straight) J2, and the number of J2 that hatched from the egg masses was recorded after 6 days. The J2 were washed from the soil extracts after 2 or 4 days by filling the wells with sterile water and collecting the supernatant after the J2 sedimentation. This was repeated four times, and after 24 h, J2 mobility was again recorded. The egg mass hatching recovery potential was tested by two transfers of each egg mass to a new well containing water, and hatching was recorded again 6 days later.

The soil extract activity on J2 was further analyzed after separating soil extracts into two fractions using Microcon 3, a 3-kDa molecular mass cut-off filter device (Amicon, Millipore Corp.). The lower molecular mass fraction (<3 kDa) was used in the bioassay as is, whereas the higher fraction (>3 kDa), which was concentrated on the membrane, was resuspended in 150 mM phosphate-buffered saline, pH 7.4, containing 5 mM $CaCl_2$. The two fractions, as well as extracts after heat treatment at 100°C for 10 min, were subjected to bioassays with J2 mobility as described previously.

Protease activity of the soil extracts. Protease activity of the different soil extracts was measured in 250 µl of a reaction mixture consisting of 50 mM Tris-HCl buffer (pH 7.5) with 5 mM $CaCl_2$ and 3 mg of azocasein (Sigma Chemical, St. Louis). The tested soil extract, or the buffer solution that served as a control (250 µl), was added to the reaction mixture and incubated for 15 min at 37°C. The reaction was stopped by adding 250 µl of 10% trichloroacetic acid. The tubes were kept for a further 30 min on ice and centrifuged at 10,000 × *g* for 10 min. The supernatant absorbance was measured at 400 nm. Protease from *Streptomyces griseus* (Sigma Chemical) was used as a standard for protease activity.

Direct interaction of *Trichoderma* T-203, WT, and P-2 with nematodes. Experiments were conducted in vitro under sterile conditions using *Trichoderma* T-203, WT, and P-2. Two-well chamber slides (Nunc Inc., Naperville, IL) were filled with a very thin layer of 1% Phytagel (Sigma Chemical). Young embryonated eggs (before the appearance of first-stage juveniles) were collected from axenic cultures, J2 were hatched from sterile eggs, and young egg masses (including the gall) were added to the chambers. Each chamber contained eggs or J2 placed near the corners in four 10-µl drops of approximately 50 eggs or J2, whereas the egg masses were each placed in a corner, four per chamber. The fungus grown on potato dextrose agar (Difco Laboratories, Detroit) plates was introduced to the center of the chamber on a small agar disk. Each life stage was tested in four chambers for each *Trichoderma* isolate and for controls of the nematode life stages without the fungus. The chambers were incubated in the dark at 27 ± 1°C, and the interaction between the fungus and the nematodes was qualitatively monitored for 1 week with an inverted microscope. Egg masses were crushed on a slide to examine the infected eggs inside.

Statistical analysis. Experiments were conducted at least twice. Analysis of variance was performed according to Tukey's honest significant difference test ($P = 0.05$).

RESULTS

Effect of *Trichoderma* T-203 preparation and controls. Tomato plants from J2-infested soils treated with the fungal peat-bran preparation T-203 exhibited a drastic reduction in root galling and a significant improvement in top fresh weight (Table 1). In soils exposed to the peat-bran carrier alone, no significant effect on galling indices was recorded. Top fresh weight of the plants was improved compared with the nematode-infected nontreated plants and was not significantly different from that of the fungal treatment (Table 1). Use of an autoclaved fungal preparation resulted in a slight reduction in the galling index and improvement in top fresh weight (Table 1). Similar experiments conducted in naturally infested soil with T-203 or WT strains gave results similar to those in the J2-infested soil (data not shown). These experiments showed that the reduction in nematode infection is mainly due to the live fungus, therefore, in subsequent experiments, we did not include the controls except nontreated soil.

Evaluation of the activity of *Trichoderma* proteinase Prb1-transformed lines. P-2 was the only line to exhibit better activity than the nontransformed WT. Galling indices recorded in the P-2 treatments were lower than with the WT (Fig. 1B), although the top fresh weight related to P-2 was not significantly different from that of the WT (Fig. 1A). The activity of P-1 was similar to that of the WT, whereas P-5 and P-6 lines exhibited even lower effects than the WT (Fig. 1B). Based on these observations, we chose P-2 for subsequent experiments.

Activity of *Trichoderma* T-203, WT, and P-2 in J2-infested soil. Top fresh weights of tomatoes planted in J2-infested soils

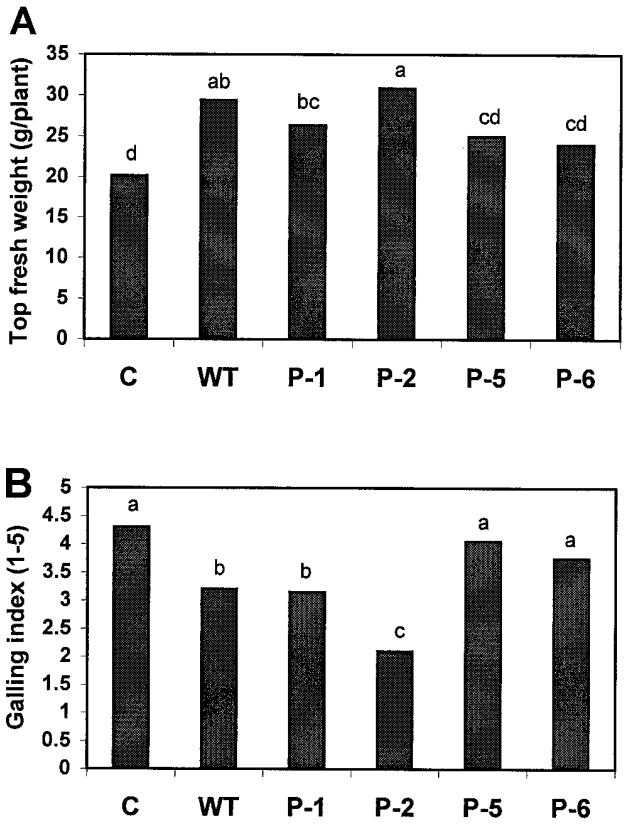

Fig. 1. Effect of *Trichoderma harzianum* proteinase Prb1-transformed strains P-1, P-2, P-5, and P-6 compared with the wild-type (WT) strain and to a nontreated control (C). *Trichoderma* peat-bran preparations of the different strains were amended to soil in 1-liter pots and inoculated with 2,000 second-stage juveniles per pot 10 days before planting tomato seedlings. One month later, **A,** top fresh weight and **B,** galling index were determined. Values are the mean of 10 replicates. Different letters above a column indicate statistical significance according to Tukey's test ($P = 0.05$).

that had been pretreated with *Trichoderma* preparations T-203, WT, or P-2 were significantly higher, whereas their galling indices were significantly lower compared with the nematode-infected nontreated plants (Table 2). The proteinase-transformed line P-2 revealed the most prominent effect (Table 2).

Evaluation of *Trichoderma* activity in naturally infested soil. In the 50-liter container experiments, tomato plants grown for 2 months in nematode-infested soil that had been pretreated for 1 month with a *Trichoderma* preparation exhibited twice the top fresh weight of nematode-infected, nontreated plants. The average root-galling index of the *Trichoderma*-treated plants was significantly lower than that of the nontreated plants (Fig. 2; Table 3). When the experiment was terminated, 7×10^5 CFU/g of soil of the fungus were counted in the *Trichoderma*-treated soil, whereas the initial population was estimated at 10^6 CFU/g of soil. *Trichoderma* spp. was not found in the control treatment.

Precolonization of the seedlings' root-ball with *Trichoderma* (T-203) during their growth in the nursery did not result in satisfactory biological control in greenhouse experiments; top fresh weight of *Trichoderma*-treated plants was significantly improved, but the nematode infection level was not significantly reduced (data not shown).

Nematode development in roots following *Trichoderma* soil treatments. Staining tomato roots that had been grown for 28 days in nematode-infested soil without *Trichoderma* strains showed that juveniles that had penetrated, had developed into adult females (Table 4), whereas in the *Trichoderma*-treated soil, fewer females were recorded in the roots and a few nematodes were still at the J4 stage, but not significantly more than in controls. No J2 or J3 were recorded in the *Trichoderma*-treated roots, indicating that the fungus had affected mostly nematode penetration into the roots rather than nematode development inside the roots (Table 4).

Effect of soil extracts on nematodes. Extracts of *Trichoderma*-treated soils incubated for 24 to 48 h with J2 affected the latter's mobility (Table 5). Soil extracts derived from the P-2-transformed line had a similar effect on J2 mobility as the soil extracts derived from the WT strain, and both extracts were more effective than that of the T-203 strain. Controls from nontreated, peat-bran treated, or autoclaved *Trichoderma* preparation soils did not inhibit J2 mobility (Table 5). When J2 were washed after 4 days from the soil extracts and incubated in water, no significant recovery was observed (data not shown); however, washing after 2 days resulted in a recovery of approximately 50% with all fungal soil treatments. Egg-mass hatching was tested only with the WT soil extract, which was very effective at inhibiting hatch-

TABLE 2. Effect of *Trichoderma harzianum* T-203, wild type (WT), and its proteinase-transformed line P-2 amended to *Meloidogyne javanica*-infested soil on tomato seedlings

Treatment[y]	Top fresh weight (gram per plant)[z]	Galling index (1 to 5 scale)[z]
T-203	78.81 b	0.93 c
WT	80.84 b	1.36 b
P-2	82.84 a	0.51 d
Nontreated soil	38.60 c	2.86 a

[y] Experiment was performed in 3-liter pots containing sandy loam soil artificially infested with 8,000 second-stage juveniles and mixed with 10 replicates of *Trichoderma* peat-bran preparation (1%, wt/wt). Ten days later, the pots were planted with tomato seedlings.

[z] Top fresh weight and galling indices of the infected roots were determined 6 weeks after planting. Values in columns followed by different letters are significantly different according to Tukey's test ($P = 0.05$).

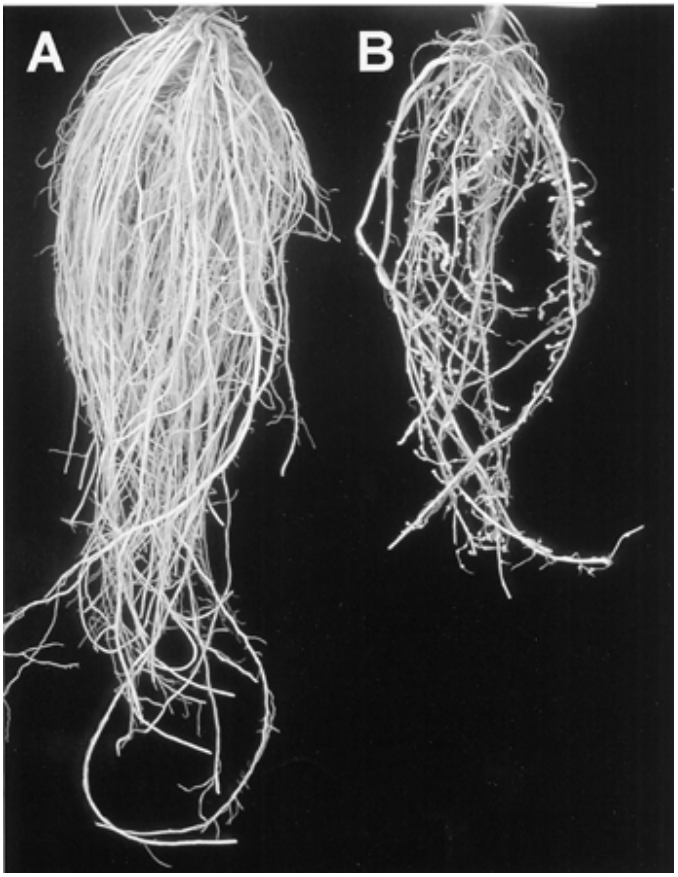

Fig. 2. Tomato roots from **A,** *Trichoderma harzianum*-treated naturally infested soil, and **B,** nontreated, naturally infested soil. Fifty-liter containers were filled with *Meloidogyne javanica* naturally infested sandy loam soil (≈2,000 second-stage juveniles per liter). One percent (wt/wt) of the fungal preparation (WT) was mixed with the soil, in the upper half of the container. Nontreated, nematode-infested soil served as a control. Four weeks after *Trichoderma* spp. application, four seedlings of tomato cv. 144 were planted in each container and grown for 2 months.

TABLE 3. Top fresh weight and galling index of tomato plants grown in *Meloidogyne javanica* naturally infested soil, treated with *Trichoderma harzianum* wild-type (WT) preparation

Treatment[y]	Top fresh weight (gram per plant)[z]	Galling index (1 to 5 scale)[z]
WT	344.27 a	0.5 b
Nontreated	169.46 b	3.5 a

[y] Experiments were conducted in 50-liter containers with naturally infested soil treated with *Trichoderma* WT peat-bran preparation (1%, wt/wt) mixed with the upper half of the soil 1 month before planting tomato seedlings. Four seedlings were planted in each container, with four containers per treatment.

[z] Top fresh weights and galling indices were determined 2 months after planting. Values in columns followed by different letters are significantly different according to Tukey's test ($P = 0.05$).

TABLE 4. *Meloidogyne javanica* developmental stages in roots, 28 days after planting tomato seedlings in *Trichoderma harzianum*-treated soil

Treatment[y]	Second-stage juveniles (J2)	Third-stage juveniles (J3)	Fourth-stage juveniles (J4)[z]	Females[z]
Wild type	0	0	2.5 a	6.5 b
T-203	0	0	0.3 a	8.3 b
P-2	0	0	2.5 a	4.3 b
Nontreated	0	0	0 a	35.5 a

[y] Experiments were conducted in 100-ml pots filled with soil infested with 100 J2 per pot, treated with *Trichoderma* peat-bran preparation (1%, wt/wt), 10 days before planting with young tomato seedlings, 10 replicates per treatment. Roots were stained with acid fuchsin to visualize the nematodes.

[z] Values in columns followed by different letters are significantly different according to Tukey's test ($P = 0.05$).

ing compared with the controls of nontreated soil (Table 5). This was not significantly changed following the transfer to water.

Characterization of soil extract activities. Protease activity levels in soil extracts derived from P-2 and WT treatments were similar, whereas that of the T-203 strain was lower by 23.3%. The proteolytic activity of all the *Trichoderma*-derived soil extracts was much higher than that of the untreated control (Table 5). Further characterization of the soil extracts' activity on J2 showed heat resistance for all *Trichoderma* strains and a molecular mass range lower than 3 kDa for the fraction that was active on the J2 (Table 6).

Direct parasitism of *Trichoderma* strains on J2, separated eggs, and egg masses. *Trichoderma* strains WT, P-2, and T-203, incubated in vitro with the nematodes, were able to coil around and penetrate the J2 (Fig. 3A). Separated young eggs were also colonized by the fungal strains (Fig. 3B), whereas mature eggs were not attacked. Egg-mass penetration was recorded only for P-2, whereas the WT and T-203 strains were not able to grow on or penetrate the egg masses and the eggs inside them.

DISCUSSION

T. harzianum strains revealed significant biocontrol activity against the root-knot nematode *M. javanica* in greenhouse experiments (Figs. 1 and 2; Tables 1 to 3). Toward improving the biocontrol process, we studied the mode of action of the fungus against this nematode.

In experiments in which control treatments of autoclaved peat-bran carrier and autoclaved fungal preparation were included, a significant effect on growth enhancement was recorded. However, their effect on root galling was nonsignificant in the case of the autoclaved peat-bran alone, whereas the autoclaved *Trichoderma* preparation resulted in a significant reduction in galling. Nevertheless, the latter always showed a significantly smaller reduction in galling index compared with the *Trichoderma* treatments. The galling reduction caused by the autoclaved preparation could have been due to heat-resistant metabolites produced by the fungus during the culturing process on the peat-bran. The autoclaved fungus could also have served as an organic amendment. Heat-resistant metabolites were found to affect the nematode's mobility and viability in our in vitro bioassays with *Trichoderma*-treated soil extracts (Tables 5 and 6). However, such activity could not be detected in soil extracts from the autoclaved preparation or in the other controls (Table 5). Our results demonstrate the necessity of using a living fungus for successful biological control in greenhouse experiments, a conclusion supported by the in vitro tests as well. Based on these results, control treatments, such as carrier alone or autoclaved fungal preparation, were not included in subsequent greenhouse experiments, except for the nontreated soil controls. The implementation of a proper control treatment as a reference in biocontrol experiments has been discussed by Baker et al. (1). Using *T. harzianum*, they demonstrated that the potential controls they had tried, such as autoclaved peat-bran or autoclaved *Trichoderma* peat-bran preparations, actually functioned as a treatment and not as a control, because their involvement resulted in a growth response.

Monitoring the developmental stages of nematodes within the roots of tomatoes planted in *Trichoderma*-treated versus nontreated soils revealed that fewer nematodes penetrate the roots in fungus-treated soil. However, no inhibition was recorded in nematode development within those roots. Preplanting application of the fungus to nematode-infested soil might enable the production of fungal metabolites with anti-nematode activity such as those found in the soil extracts, which could immobilize J2 and thus

TABLE 5. Soil extracts from *Trichoderma harzianum*-treated soils, protease activity, and in vitro effect on *Meloidogyne javanica* second-stage juvenile (J2) mobility and egg-mass hatching[w]

Treatments	% J2 immobility[x]	No. of J2 hatched per egg mass[y]	Protease activity (unit/ml)[z]
Wild type (WT)	92.3 a	4.2 b	0.44 a
P-2	95.2 a	nt	0.42 a
T-203	75.2 b	nt	0.33 b
Nontreated soil	2.4 c	132.4 a	0.12 c
Peat-bran control	2.7 c	125.0 a	nt
Autoclaved WT	3.1 c	nt	nt
Water	1.2 c	141.5 a	…

[w] Values in columns followed by different letters are significantly different according to Tukey's test ($P = 0.05$). nt = not tested.
[x] J2 (≈100) in five replicates were incubated in 96 multiwell flat-bottom plates with soil extracts for 48 h at 25 ± 2°C.
[y] One egg mass per well, in 10 replicates, was incubated with soil extracts as described for J2 and hatching was counted after 6 days.
[z] Protease activity was tested with azocasein as a substrate. One unit hydrolyzes casein to produce 1 μm of tyrosine per minute at pH 7.5 at 37°C.

TABLE 6. Effect of extracts from *Trichoderma*-treated soils on mobility of *Meloidogyne javanica* second-stage juveniles (J2)

Treatments	% J2 immobility[x]		
	Wild type	P-2	T-203
Soil extract	90.5 a	94.5 a	72.5 b
Boiled soil extract[y]	91.0 a	93.0 a	75.0 b
Fraction <3 kDa[z]	93.0 a	93.0 a	73.4 b
Fraction >3 kDa[z]	2.5 c	3.0 c	1.5 c
Water	2.3 c	…	…

[x] J2 (≈100) in five replicates were incubated in 96 multiwell flat-bottom plates with treated or nontreated soil extracts for 48 h at 25 ± 2°C. Values followed by different letters are significantly different according to Tukey's test ($P = 0.05$).
[y] Soil extracts were boiled in water for 10 min.
[z] A 3-kDa molecular mass cut-off filter device was used. The lower molecular mass fraction (<3 kDa) was used in the bioassay as is, whereas the higher fraction (>3 kDa), which was concentrated on the membrane, was resuspended in 150 mM phosphate-buffered saline, pH 7.4, containing 5 mM $CaCl_2$.

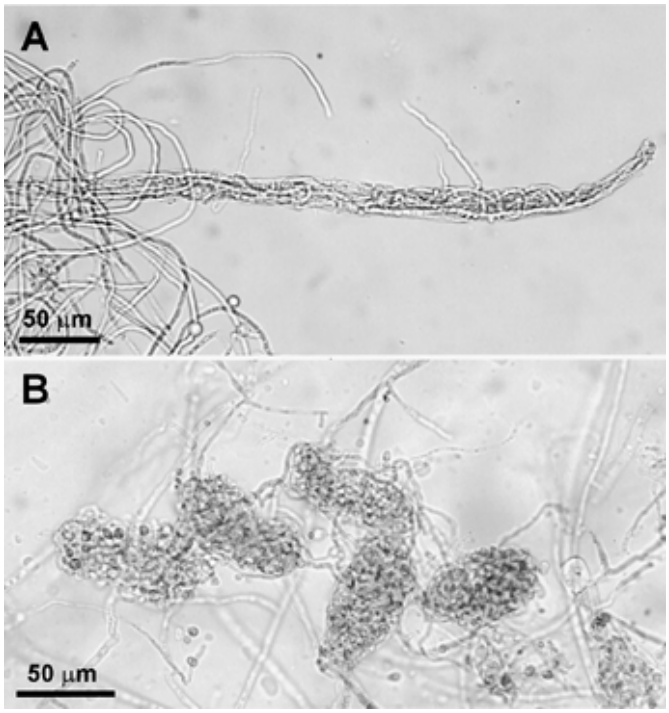

Fig. 3. Direct parasitism of *Trichoderma harzianum* (T-203) on *Meloidogyne javanica*. **A,** Second-stage juveniles and **B,** young eggs. Experiments were conducted in vitro under sterile conditions on 1% Phytagel. Eggs were collected from axenic cultures with 0.5% hypochlorite, and juveniles were hatched from the eggs.

reduce nematode penetration. Moreover, via soil application, the fungus might directly parasitize the nematodes. When naturally infested soil was used, a longer preplanting incubation period was required to achieve significant control compared with J2-infested soil. This might be due to immediate exposure of J2 to the fungus and its metabolites in J2-infested soil, whereas in naturally infested soil, where gradual hatching of the eggs takes place, longer exposure to the *Trichoderma* preparation is required.

The J2 cuticle is composed mainly of proteins (4); therefore, we assumed that improved proteolytic activity of the antagonist would lead to increased biocontrol ability. Proteinase Prb1-transformed lines that had been previously tested for their efficiency in fungal biological control (15) were used. Of these, only line P-2 was more effective than the WT strain in reducing galling index (Fig. 1B), whereas three of the transformed lines (P-2, P-5, and P-6) showed significantly improved biocontrol ability against the fungal pathogen *R. solani* on cotton compared with the WT, with P-2 showing the highest activity (15). The differences between the transformed strains, expressed by *prb1* mRNA production levels, Prb1 protein production, protease activity, and growth rates, are discussed by Flores et al. (15). They concluded that although line P-2 exhibits intermediate levels of proteinase production and activity compared with the other transformants, its growth rate is less reduced by the transformation process. This could explain why lines P-5 and P-6 showed reduced rather than improved capacity to control nematodes in our experiment.

WT and P-2 soil extracts revealed the same nematicidal and proteolytic activities (Table 5), significantly higher than that of strain T-203 (Table 5). The active fraction on J2 was of low molecular mass (less than 3 kDa) and heat resistant (Table 6). Although there was a correlation between the general proteolytic activity of the soil extracts and their effect on J2, the proteases present in the soil extracts probably did not directly affect the J2.

Prb1 is an inducible proteinase, expressed after induction by the presence of host-fungal materials, such as cell walls or chitin, and repressed by glucose (16). Cortes et al. (8) showed that the gene expression is triggered by a diffusible factor, and does not require direct contact with the host. This gene might also be expressed during direct interaction between the fungus and the nematode's different life stages. In in vitro assays for direct interaction, all the strains attacked J2 and separated eggs, whereas P-2 was the only strain that had the ability to penetrate egg masses and colonize the eggs compared with the WT and T-203 strains.

It is suggested that the main antinematode activity caused by *T. harzianum* takes place in the soil and not within the roots. An induced-resistance cascade can probably be excluded, because application of the *Trichoderma* preparation to the root system alone did not result in sufficient biological control, nor did it prevent nematode penetration or development inside the roots. This work supports the hypothesis that improved proteolytic activity of *Trichoderma* strains is important for the nematode biocontrol process in both suggested mechanisms: effect of metabolites produced by the fungus in the soil and direct parasitism by the antagonist.

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