Trichoderma strains as growth promoters in *Capsicum annuum* and as biocontrol agents in *Meloidogyne incognita*

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ABSTRACT

The *Trichoderma* species act as plant symbionts, decomposers of organic material, and as antagonists of root phytoparasites. To estimate the potential of four native strains of *Trichoderma* as growth promoters of *Capsicum annuum* L. and as biocontrol against root knot nematode *Meloidogyne incognita* (Kofoid and White) Chitwood, two bioassays in greenhouse were established. In one, the capacity of *Trichoderma* to promote initial growth of seedlings (36 d after planting, dap) was evaluated. In the other, the biocontrol of *Trichoderma* against *M. incognita* at 96 dap was also evaluated. The *Trichoderma* strains and the chemical fertilization treatment significantly promoted seedling growth at 36 dap ($P \leq 0.01$). With respect to the control, *T. atroviride* promoted greater height in the seedlings, while *T. atroviride*, *T. virens* and *T. harzianum*-C2 increased fresh weight in roots (60.14%) and both *T. atroviride* and *T. harzianum*-C2 produced up to 82.30% more dry root biomass. In biocontrol, the control plants registered the highest damage with galling indexes of 85.50% ($P \leq 0.01$). The lowest galling indexes were estimated with all the *Trichoderma* strains (21.60% to 35%). *Trichoderma atroviride* reduced egg production by 63% and the production of females by 14.36%, with respect to the oxamyl nematicide. Biocontrol of the nematode through the application of the *Trichoderma* strains favored growth of the plants in general. The fungal species studied show potential as growth promoting agents and as biocontrol agents *M. incognita* in *C. annuum*.

Key words: Antagonistic fungi, interaction, root-knot nematodes, sweet pepper.

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INTRODUCTION

The fungi of the genus *Trichoderma*, present in all the soils, are cosmopolitan and can be found in different climate zones all over the world. They are generally decomposers of ligneous and herbaceous material, but can also be plant symbionts (Hoyos-Carvajal and Bissett, 2011; Zhang et al., 2015) and promote their growth thanks to the production of growth regulators, organic acids and siderophores (Contreras-Cornejo et al., 2009; Aguado-Santacruz et al., 2012; Hermosa et al., 2013). Moreover, the *Trichoderma* species can act as biological control agents, in particular against nematodes of the genus *Meloidogyne* (Candelero et al., 2015; Sokhandani et al., 2016), by producing enzymes and secondary metabolites (Bhattacharjee and Dey, 2014), which inhibit egg hatching and immobilize second stage juveniles (J_2) of the nematode (Feyisa et al., 2015). The capacity to control populations of root phytopathogens is associated with the origin of the fungus, with the compatible interaction of the host plants and the edaphic conditions of their location (Affokpon et al., 2011; Al-Hazmi and TariqJaveed, 2016).

On a global level, the losses caused by *Meloidogyne* spp. in crop production can be estimated at 100 million dollars (Oka, 2010). In Mexico, *M. incognita* is distributed all over the country and in the southern region it is associated with at least 17 plant species (Herrera et al., 2011), among which *Capsicum annuum* L. is of particular importance. As a control tactic of *M. incognita*, chemical nematicides are applied, however, in the cultivation of *C. annuum*, their use has been restricted due to their toxicity levels, residual elements in the environment and selection of populations of resistant nematodes (Xie et al., 2015); this has stimulated studies in which the tendency has been to find non contaminating and environmentally friendly alternatives for the ecosystems.

Reports are available on the use of beneficial microorganisms from the soil to control the nematodes; however, in Mexico, very few studies have explored the investigation of microorganisms, which promote growth of the crops and biocontrol of galling nematodes (Candelero et al., 2015). The activity of the native *Trichoderma* strains and the effect they may have on the pathosystem *C. annuum-M. incognita* is unknown. The objectives of this work were to estimate, under greenhouse conditions, the effect of four native *Trichoderma* strains on the initial growth of *C. annuum*, and their capacity to reduce the galling index, reproduction and the damage caused by *M. incognita*.

MATERIALS AND METHODS

Origin of *Trichoderma* spp.

The *Trichoderma* strains were obtained in the state of Yucatan, from soil in sites with no agricultural activity over the last 30 yr; these sites are located in Tizimin (21°09'29.1" N, 88°10'26.8" W), Oxkutzkab (20°18'11" N, 89°21'46.5" W), and San Felipe (21°32'33.8" N, 88°14'20.2" W). For their isolation, the washing and particle filtration technique proposed by Bills et al. (2007) was used and they were preserved in a potato dextrose agar culture medium (DIBICO, S.A. de C.V., México) and in mineral oil. The *Trichoderma* species were molecularly identified, based on the National Center for Biotechnology Information (NCBI, Bethesda, Maryland, USA), as *T. harzianum*-C1 (KJ028794), *T. atroviride* (HMO47766), *T. virens* (KF144629) and *T. harzianum*-C2 (KF201995). These species were selected because they presented antagonistic activity *in vitro* against *M. incognita* in a previous study (Candelero et al., 2015).

Evaluation of *Trichoderma* in *C. annuum* seedbed

The *Trichoderma* strains conserved in mineral oil were reactivated in a potato dextrose agar culture medium (DIBICO, S.A. de C.V., Mexico). Once growth of the fungi was obtained, concentrations of 1×10^6 spores mL^{-1} were prepared according to Cubillos-Hinojosa et al. (2009). Seeds of *C. annuum* 'Criollo' were disinfested with sodium hypochlorite at 1% for 2 min, followed by two rinses with sterile distilled water, after which they were planted in trays containing a sterile commercial substrate (Sunshine, Sun Gro Horticulture, Agawam, Massachusetts, USA). Four inoculations were carried out with the spore concentration indicated in the seeds at the moment of planting and in the roots of seedlings at 10, 20 and 30 d after germination. Irrigation was performed according to the water needs of the seedlings.

Six treatments were evaluated: Plants inoculated with *T. harzianum*-C1, *T. atroviride*, *T. virens*, *T. harzianum*-C2, Control (only water, without *Trichoderma* and without fertilization), and chemical fertilization (Poly-Feed, 17-17-17, Haifa Chemicals, Mexico) without *Trichoderma*, applied two times per week along with the irrigations. Each treatment consisted of 10 replicates (with five plants as an experimental unit) distributed in a completely random experimental design. The plants were maintained under greenhouse conditions at 28 ± 2 °C, 64% RH, and light intensity of 450 lux, on average, for 36 d. Thirty-six days after planting (dap), growth variables were estimated: Plant height, dry shoot biomass, root fresh weight, and root dry weight.

Biocontrol experiment

Procurement of J2 of *M. incognita.* In Yucatan, Mexico, samples were collected from commercial plantations of *C. annuum* 'Criollo', naturally parasitized by *M. incognita*. The parasitized and galled roots were deposited in paper bags and conserved at 6 °C refrigeration for 24 h. They were then washed under running water and egg masses were extracted with syringes under stereo microscope (Leica M80, Leica Microsystems, Buffalo Grove, Illinois, USA). These were disinfected with sodium hypochlorite at 1% for 2 min and rinsed with sterile distilled water until the disinfectant was eliminated. The eggs were incubated at 25 ± 1 °C for 3 d until J2 hatching. Subsequently, they were concentrated in a 500 mL flask; 1 mL was taken and they were counted in order to calibrate the inoculum. From the roots with galls, adult females were extracted to identify species by means of perineal cuts (Ayoub, 1977).

Evaluation of *Trichoderma* as antagonists of *M. incognita.* To estimate the level of antagonism exerted by *Trichoderma* strains on *M. incognita*, soil was sterilized by steam dragging over a period of 3 d for 1 h at 90 °C; the sterile substrate was then used to fill 2 kg plastic pots. Before transplanting, a hole was prepared, 3 cm in diameter and 5 cm deep into which 1 mL water containing 1000 larvae eggs and 300 J_2 of *M. incognita* was inoculated, followed by the immediate transplant of a 36 d old *C. annuum* plant inoculated with the *Trichoderma* strains, as previously described. The nutritional management of the plants consisted of fertilization with chemical balance 2:1:1 with: urea (Magro, 46-00-00, Fertinova, Mexico), potassium nitrate (Ultrasol, 12-00-46, SQM, Mexico) and monoammonium phosphate (MAP, 12-61-00 Greenhow S. A. de C.V., México), applied two times a week throughout the experiment.

Six treatments were evaluated comprising four strains of *Trichoderma* (*Trichoderma harzianum*-C1, *T. atroviride*, *T. virens* and *T. harzianum*-C2), an oxamyl ([*EZ*]-*N*,*N*-dimethyl-2-methylcarbamoyloxyimino-2-[methylthioacetamide) nematicide 24% in doses of 1 mL L^{-1} water (Vydate, Dupont, Mexico), applied to the soil at the moment of transplant, and a control with nematodes only. Each treatment consisted of 10 plants (replicates) which constituted the replicates and the experimental units, distributed in homogenous conditions in a completely random experimental design and maintained in greenhouse conditions at 28 ± 2 °C, 64% RH, and light intensity of 450 lux on average.

The treatments were evaluated after 96 dap and damage variables were determined as the number of galls per root and the galling index according to the scale of radical damage proposed by Taylor and Sasser (1983): 0 = healthy radical system, without infection, 1 = 1% to 10% radical system presents small galls, 2 = 11% to 25% radical system is severely galled, 3 = 26% to 50% radical system is severely galled, 4 = 51% to 75% radical system is severely galled, and 5 = 76% to 100% radical system is severely galled, healthy roots are scarce.

The variables of nematode reproduction estimated were the number of eggs per gram of root and the number of females per gram of root. To obtain this, the root of each plant was fragmented and subsequently homogenized; 2 g were taken, 1 g was liquefied for 11 s with sodium

hypochlorite at 2% to obtain eggs, which were then counted under a microscope (4X; Leica, USA), the other was stained with acid fuchsine at l% at boiling point for 10 min. Once the roots were stained, they were deposited in vials with glycerin at 78% for the subsequent dissection and counting of adult females under a stereo microscope (Leica M80). Growth variables were also evaluated: Plant height, dry shoot biomass, fresh weight, and long of the root.

Statistical analysis of data

One way ANOVA was performed, and in the case of galling index data that not comply with the suppositions of normal distribution and homogeneity of variances, these were transformed by means of the arcsine function [y = arcsin (sqrt x/100)]. As a comparison of averages, the method of Tukey ($P = 0.05$) was used with the statistical package Statistical Analysis System, version 9.3 (SAS Institute, Cary, North Carolina, USA).

RESULTS AND DISCUSSION

Effect of *Trichoderma* on *C. annuum* seedbed

Trichoderma strains promoted significant growth ($P \leq 0.01$) in *C. annuum* seedlings at 36 dap in comparison with control seedlings (without inoculation with *Trichoderma* and without fertilization). In particular, *T. atroviride* and chemical fertilization treatment increased plant height by 17.28% and 22.68%, with respect to control plants. The production of dry shoot biomass was significantly ($P \leq 0.01$) increased with the treatments that included *Trichoderma* and chemical fertilization; obtaining gains up to 47.61% compared to the control (Table 1). Root fresh weight increased significantly ($P \leq 0.01$) with chemical fertilization and with *T. atroviride*, *T. virens* and *T. harzianum*-C2 up to 60.14%, in comparison with the control. Finally, a significant effect on dry root biomass was observed in the treatments with *T. atroviride* and chemical fertilization, with increases of 82.30% higher than the control (Table 1).

Treatments including *Trichoderma* strains produced seedlings similar to those obtained with the chemical fertilization; healthy, vigorous and with the development of roots, desirable characteristics that allow a greater probability of establishment and survival under the prevailing conditions in their designated sites of cultivation. These parameters have been reported in *C. annuum* and *C. chinense* when the use of *Trichoderma* is implemented (Deshmukh et al., 2012; Candelero et al., 2015). This growth promoting effect in seedlings was also reported in *C. annuum* with *T. virens*-TVC_3, where the seedlings presented greater height (69.76%) and longer roots (27.17%) (Muthukumar et al., 2011), while *T. harzianum* promoted higher fresh (29.05%) and dry shoot biomass (53.92%) (Deshmukh et al., 2012). In vegetables such as *Lactuca sativa*, *Trichoderma* spp. strains favored DM production (89%) and in *Rhaphanus sativus* the production of larger tubers (79%) (Ortuño et al., 2013). In *C. chinense*, *T. harzianum* strain increased the radical volume (84.61%) in comparison with seedlings without fungus (Candelero et al., 2015).

The growth promoting effect induced by the *Trichoderma* species is associated with their capacity to produce secondary metabolites such as indole-acetic acid (Contreras-Cornejo et al., 2009), a hormone that induces growth in meristematic tissues, and increases the production of cysteine (QID74) which modifies root architecture and increases their growth (Samolski et al., 2012). The production of organic acids such as gluconic, citric and fumaric acids, which lower the pH of soil solution and allow solubilization of phosphates, micronutrients and mineral cations such as Fe, Mn and Mg, which are useful for plant metabolism (Hermosa et al., 2013).

In Mexico, *Capsicum* spp. seedlings are produced every year to be established in 21 555 ha for cultivation (SIAP, 2016). The production of seedlings involves the indiscriminate use of chemical fertilizers (Pérez-Olvera et al., 2011). In this study, the application of *Trichoderma* strains generated *C. annuum* seedlings with characteristic similar to those of plants treated with chemical fertilization, demonstrating the advantage of using these microorganisms as a complementary alternative for the production of *C. annuum* seedlings, which can lower economic and environmental costs by reducing the use of chemical compounds for fertilization.

Effect of *Trichoderma* strains on *M. incognita*

The control plants inoculated with *M. incognita*, without *Trichoderma* spp. and without application of oxamyl, were

Table 1. Growth variables estimated in *Capsicum annuum* seedlings inoculated with *Trichoderma* strains at 36 d after planting in the different treatments.

Treatments	Plant height	Dry shoot biomass	Fresh root weight	Dry root weight
	cm	g		
T. harzianum-C1	12.40 ± 1.03b	0.144 ± 0.015a	1.06 ± 0.26b	0.070 ± 0.011c
T. atroviride	13.02 ± 0.71ab	0.164 ± 0.015a	1.38 ± 0.15a	0.111 ± 0.008a
T. virens	12.77 ± 0.55b	0.146 ± 0.028a	1.23 ± 0.27ab	0.066 ± 0.009c
T. harzianum-C2	12.72 ± 0.54b	0.157 ± 0.026a	1.25 ± 0.22ab	0.085 ± 0.011b
Chemical fertilization	13.93 ± 0.70a	0.168 ± 0.010a	1.38 ± 0.70a	0.113 ± 0.006a
Control	10.77 ± 0.92c	0.088 ± 0.007b	0.55 ± 0.07c	0.020 ± 0.006d
LSD	1.10	0.027	0.25	0.013

The table shows average ± standard deviation. N = 60.
LSD: Least significant difference.
Values with different letters in a column indicate significant differences according to Tukey's test ($P = 0.05$).

susceptible to the nematode. It was possible to observe destroyed roots (Figure 2), which implies a smaller number of galls, but a higher galling index (85.50%), as well as a smaller number of eggs (1441 ± 91.14) and females (31.40 ± 4.32) ($P \leq 0.01$) (Table 2). The nematode induces alterations in the continuity of the vascular tissue, destroying the root and limiting the spaces available for new infestations, resulting in a reduction of feeding sites and a diminished capacity of reproduction for the nematode (Medina-Canales et al., 2011; Hernández-Ochandía et al., 2012). This susceptibility to *M. incognita* has already been reported in *C. annuum* 'Sweet Mini Pepper' (Aguilar et al., 2014) 'Baron' and 'Atlante' (Ros-Ibáñez et al., 2014), where galling indexes above 50% were estimated, with destroyed roots, chlorotic plants, and reduction in growth, as can be observed in control plants presented in this study (Figures 1 and 2).

With respect to the biocontrol presented by *Trichoderma* against *M. incognita*, significant effects were obtained ($P \leq 0.01$) in the reduction of the number of galls per root, caused by the *Trichoderma* strains and oxamyl; in particular, *T. atroviride* was 20.11% more effective than oxamyl. The other *Trichoderma* strains were equally effective, with respect to oxamyl treatment. The reduced number of galls as a result of these fungi has also been reported in *Cucumis sativus* plants where *T. virens* reduced the number of galls by 50% (Yankova et al., 2014) and in *Solanum lycopersicum* with *T. harzianum* by 75% (Pinzón et al., 2015).

The galling index was associated with size and distribution of galls on the root, this was significantly lower ($P \leq 0.01$) with the inoculation of the *Trichoderma* strains, with which reductions of up to 55.46% were estimated in relation to the oxamyl and up to 74.73% with respect to the control (Table 2). The results indicated a better capacity of biocontrol of *M. incognita* with the *Trichoderma* strains in comparison with oxamyl. Their effect was similar to those reported with other native strains such as *T. virens* against *Meloidogyne* spp. in *C. sativus*, where their application reduced the galling index by 50% (Zhang et al., 2013).

The application of *T. atroviride* significantly reduced ($P \leq 0.01$) the number of eggs and females per gram of root, with reductions of 26.71% and 21.90%, respectively, with respect to oxamyl (Table 2). *Trichoderma harzianum*-C2 and *T. harzianum*-C1 also limited reproduction of the nematode and their antagonism was greater than oxamyl, with reductions in egg production of 17.67% and 9.18% and formation of females of 14.52% and 8.80%. In the case of *T. virens*, although a galling index similar to the other strains was registered (24.75%), a larger number of eggs and females per gram of root were produced, up to 18% and 11%, respectively, more than was registered with the oxamyl. This result is due to the fact that *T. virens* promoted greater root growth, resulting in the availability of more spaces for nematode feeding and new infections of J_2 to form new galls, giving rise to a greater quantity of eggs and females (Tables 2 and 3).

The capacity to inhibit the reproduction of the nematode with the application of *Trichoderma* spp. strains was reported in *C. annuum* with *T. virens*, where egg formation was reduced by 60% (Meyer et al., 2001) and in *S. lycopersicum* the strains *T. brevicompactum* and *T. longibrachiatum* suppressed egg production by 86% and 84%, respectively (Affokpon et al., 2011; Zhang et al., 2015). In this same plant species, *T. harzianum* prevented the production of females up to 86% (Pinzón et al., 2015). The biocontrol mechanisms of *Trichoderma* against nematodes may be associated with the production of lysis enzymes such as glucanases, chitinases, and proteases that affect cuticle and viability of nematode eggs (Szabó et al., 2012; Zhang et al., 2015); they may also be implicated in the activation of plant defense mechanisms when *Trichoderma* spp. establish an endophyte interaction with them (Hermosa et al., 2013; Zhang et al., 2013).

The reduction in the number of galls and in root damage together with the suppression of the nematode population by the *Trichoderma* strains promoted growth in the plants. With respect to the control plants, growth was significantly lower ($P \leq 0.01$) in comparison with those treated with *Trichoderma* spp. and the oxamyl (Figures 1 and 2). Maximum height increase in the plants was obtained with *T. harzianum*-C1 and *T. atroviride*, which were 50.91% and 52.75% higher than control plants (Table 3).

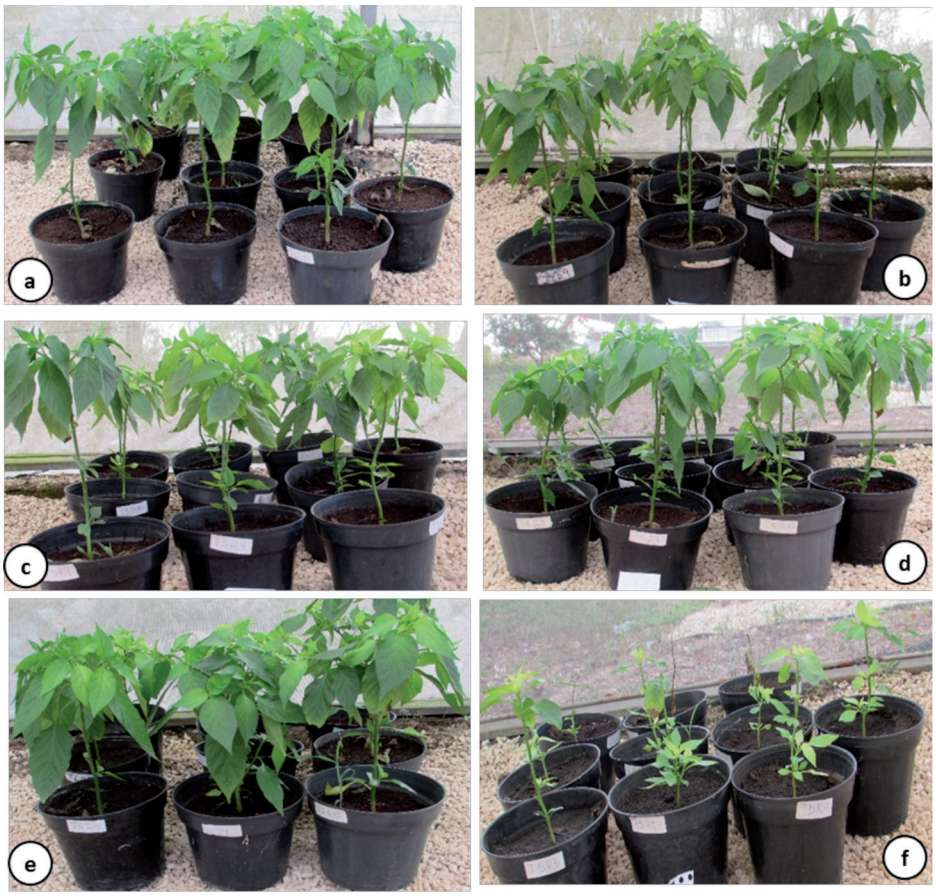
The highest dry shoot biomass was estimated with *T. harzianum*-C1 (3.03 g) and with the oxamyl (2.62 g); with the other *Trichoderma* strains, increases higher than the control were registered, up to 70.92%. With oxamyl and *Trichoderma* strains, a significant growth of the root was

Table 2. Reproduction variables of *Meloidogyne incognita* estimated at 96 d after their inoculation in *Capsicum annuum* plants in the different treatments.

Treatments	Number galls per root	Galling index (%)	Number eggs per g root	Number females per g root
Trichoderma harzianum-C1	88.00 ± 15.41a	32.00 ± 9.66c	2502 ± 131.82c	38.30 ± 3.46bc
T. atroviride	69.50 ± 6.75b	35.00 ± 17.51c	2019 ± 125.66e	32.80 ± 0.78d
T. virens	73.30 ± 7.02ab	24.75 ± 12.02c	3249 ± 125.87a	47.10 ± 5.89a
T. harzianum-C2	84.40 ± 11.15a	21.60 ± 7.58c	2268 ± 249.36d	35.90 ± 2.76cd
Oxamyl	87.00 ± 7.91a	48.50 ± 16.40b	2755 ± 156.30b	42.00 ± 4.29ab
Control	44.80 ± 15.29c	85.50 ± 7.90a	1441 ± 91.14f	14.00 ± 4.32e
LSD	14.8	14.50	228.28	5.17

The table shows average ± standard deviation. N = 60.
LSD: Least significant difference.
Values with different letters in a column indicate significant differences according to Tukey's test ($P = 0.05$).
Oxamyl 24% applied at doses 1 mL L^{-1} water.

Figure 1. *Capsicum annuum* plants inoculated with *Meloidogyne incognita* at 96 d after planting in different treatments: a) *Trichoderma harzianum*-C1, b) *T. atroviride*, c) *T. virens*, d) *T. harzianum*-C2, e) oxamyl, and f) control.



obtained ($P \leq 0.01$). In relation to the control, oxamyl and *T. virens* registered the highest root fresh weights with 84.47% and 87% respectively; moreover, with these same treatments, including *T. harzianum*-C2, roots up to 59% longer were obtained (Table 3). The favorable effect of *Trichoderma* spp. on the growth of plants parasitized by *M. incognita* has also been observed in *S. lycopersicum* (Yan et al., 2011; Radwan et al., 2012) and *C. sativus* (Pinzón et al., 2015). The application of inoculants such as *Trichoderma* has the capacity to reduce the population of this nematode and to improve the functionality of the radical system of the plants.

CONCLUSIONS

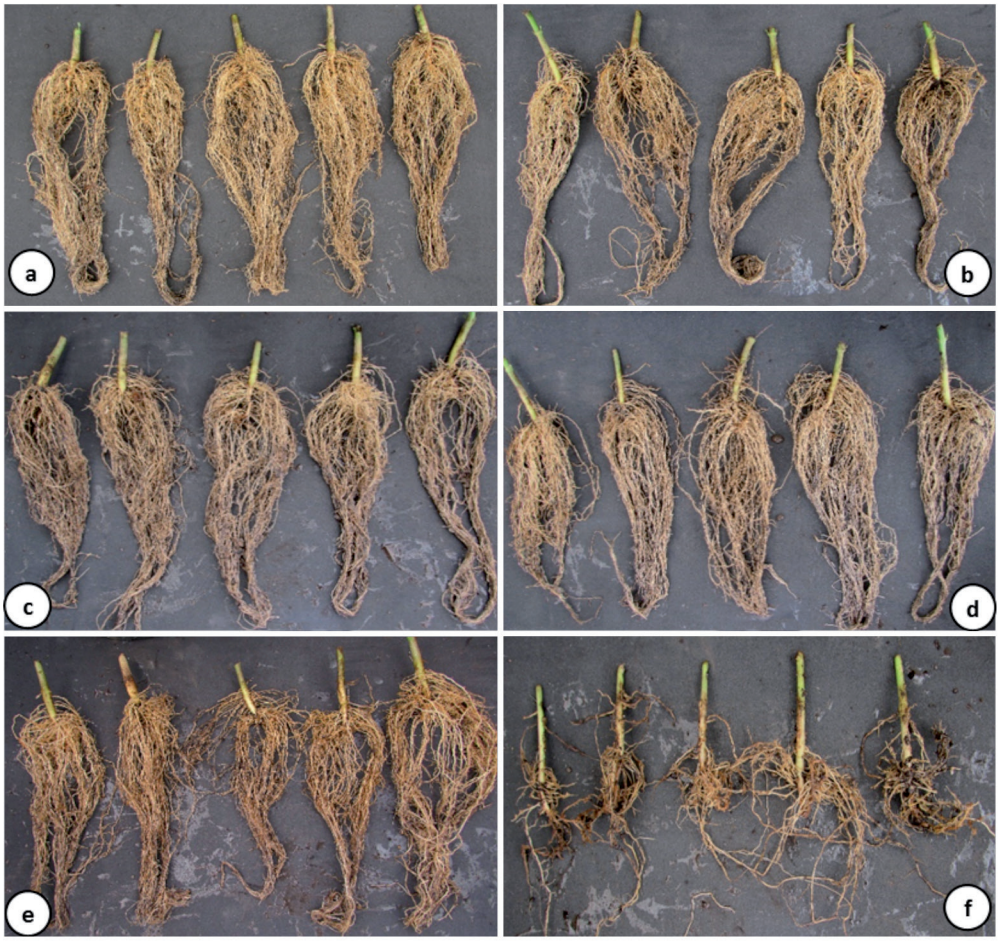
The native strains of *Trichoderma* promoted initial growth of the *Capsicum annuum* seedlings; the *Trichoderma atroviride* strain, in particular, was the most efficient in promoting general growth of the seedlings and also showed the best response in the biocontrol of *Meloidogyne incognita*, surpassing the effect of the oxamyl. The use of native strains of *Trichoderma* is a viable alternative to the excessive application of fertilizers and nematicides, and will contribute to a reduction in environmental contamination while promoting the use of microbial resources.

Table 3. Growth variables estimated in *Capsicum annuum* inoculated with *Meloidogyne incognita* at 96 d after planting in the different treatments.

Treatments	Plant height	Dry shoot biomass	Fresh root weight	Long root
	cm	g		cm
Trichoderma harzianum-C1	31.20 ± 3.29a	3.03 ± 0.28a	8.31 ± 1.64bc	25.80 ± 3.08c
T. atroviridae	30.05 ± 1.92a	2.22 ± 0.34bc	7.80 ± 1.80bc	30.70 ± 3.16ab
T. virens	25.60 ± 2.50b	1.74 ± 0.30c	9.92 ± 2.60ab	34.90 ± 3.07a
T. harzianum-C2	27.80 ± 2.40ab	2.27 ± 0.67bc	7.36 ± 1.21c	30.30 ± 3.05b
Oxamyl	25.80 ± 2.61b	2.62 ± 0.50ab	11.55 ± 1.97a	31.30 ± 3.56ab
Control	14.75 ± 2.61c	0.66 ± 0.19d	1.54 ± 0.48d	14.40 ± 3.68d
LSD	3.44	0.55	2.31	4.33

The table shows average ± standard deviation. N = 60. LSD: Least significant difference.
Values with different letters in a column indicate significant differences according to Tukey's test ($P = 0.05$).
Oxamyl 24% applied at doses of 1 mL L^{-1} water.

Figure 2. ***Capsicum annuum*** **roots inoculated with** ***Meloidogyne incognita*** **at 96 d after planting in the different treatments: a)** ***Trichoderma harzianum*****-C1, b)** ***T. atroviride*****, c)** ***T. virens*****, d)** ***T. harzianum*****-C2, e) oxamyl, and f) control.**



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