Species of *Trichoderma* antagonistic to the root knot nematode (*Meloidogyne incognita*) in habanero pepper

Especies de *Trichoderma* antagónicas al nematodo agallador (*Meloidogyne incognita*) en chile habanero

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**Abstract.** The root knot nematode *Meloidogyne* spp. is an important endoparasite limiting the cultivation of horticultural species and affecting the plants of at least 3000 species, including grasses, vegetables, ornamentals, fruit and forest. The aim of this study was to estimate the potential of three species of *Trichoderma* as antagonists of *M. incognita* in plants of *Capsicum chinense* under greenhouse conditions. A bioassay was established with plants of *C. chinense* previously inoculated with *Trichoderma* spp. and transplanted to pots with sterile substrate; the plants were subsequently inoculated with 300 infective second-stage larvae (J2) and 1000 larvae eggs of *M. incognita*. A completely randomized experimental design was implemented with six treatments (T. harzianum-C1, T. atroviride, T. virens, T. harzianum-C2, nematicide oxamyl 24% SL and a control) with 10 replicates for each treatment. After 60 days, the following parameters were recorded: number of galls per root, galling index, number of eggs and number of females per gram of root, as well as, plant height, dry shoot biomass, volume, length and fresh weight of root. In comparison with the control, the plants inoculated with the *Trichoderma* species and those which received the application of the nematicide oxamyl presented a reduction of 68.18% and 65.07%, respectively, in the number of galls per root, a galling index of 82.91% and 70.25%, respectively, number of eggs per gram of root 82.69% and 77.16%, respectively, and number of females per gram of root up to 50%. The fungal species studied in this work have potential as antagonistic agents against *M. incognita*, given that they present the same capacity as the nematicide oxamyl to reduce the galling index and reproduction of the nematode.

**Keywords:** Biological control; *Capsicum chinense*; Galling index; *M. incognita*.

**Resumen.** El nematodo agallador *Meloidogyne* spp. es un endoparásito importante que limita el cultivo de especies hortícolas, afecta al menos 3000 especies de plantas que incluyen pastos, hortalizas, ornamentales, frutales y forestales. El objetivo de este trabajo consistió en estimar el potencial de tres especies de *Trichoderma* como antagonistas de *M. incognita* en plantas de *Capsicum chinense* en condiciones de invernadero. Se estableció un bioensayo con plantas de *C. chinense* inoculadas previamente con *Trichoderma* spp. y se trasplantaron con macetas con sustrato estéril, posteriormente se inocularon con 300 larvas infectivas de segundo estadío (J2) y 1000 huevos larvados de *M. incognita*, se empleó un diseño experimental completamente al azar con seis tratamientos (T. harzianum-C1, T. atroviride, T. virens, T. harzianum-C2, nematicida oxamíl al 24% SL y un control) y 10 repeticiones por cada tratamiento. Transcurridos 60 días se estimaron el número de agallas por raíz, el índice de agallamiento, el número de huevos y el número de hembras por gramo de raíz, así como, la altura de planta, la biomasa seca de la parte aérea, el volumen, largo y peso fresco de la raíz. Las plantas inoculadas con las especies de *Trichoderma* y la aplicación del nematicida oxamíl disminuyeron en relación al control un 68,18% y 65,07% el número de agallas por raíz, un 82,91% y 70,25% el índice de agallamiento, un 82,69% y 77,16% el número de huevos por gramo de raíz y hasta un 50% el número de hembras por gramo de raíz. Las especies fúngicas estudiadas tienen potencial como agentes antagónicos contra *M. incognita* ya que presentaron la misma capacidad que el nematicida oxamíl para reducir el índice de agallamiento y la reproducción del nematodo.

**Palabras clave:** Control biológico; *Capsicum chinense*, Índice de agallamiento; *M. incognita*.
INTRODUCTION

The root knot nematode Meloidogyne spp. is an important endoparasite limiting the cultivation of horticultural species and affecting the species of at least 3000 plants, including grasses, vegetables, ornamentals, fruits and forests (Ramírez, 2014; Tovar, 2014). It can cause yield losses ranging from 18 to 100%, depending on the crop that it parasitizes (Herrera et al., 2011; Medina et al., 2011, Medina et al., 2012). M. incognita (Kofoid & White) Chitwood affects crops of commercial interest in the southeast area of Mexico, such as the chili pepper (Capsicum chinense Jacq.). Its management is based on the application of organophosphates and carbamate nematicides (Marbán & Manzanilla, 2012). Due to their toxicity and persistence, the tendency is to assess control alternatives that are compatible with both the environment and the health of agro-ecosystems (Xie et al., 2015). An example of this is the incorporation of antagonistic fungi, such as Trichoderma spp. which have the ability to parasitize nematode stages (Szabó et al., 2012, Szabó et al., 2013), producing secondary metabolites, as well as viridine, gliotoxin, gliovirine, peptaibols, trichodermin, suzukaciline and alameticine antibiotics, which inhibit egg hatching and immobilize J2 (Candelero et al., 2015; Feyisa et al., 2015) as well as chitinases, glucanases, peroxidases and chitobiose enzymes that hydrolyze the nematode cuticle components (Jiménez et al., 2013; Bhattacharjee & Dey, 2014).

The compounds mentioned above are considered to be the main antagonism mechanisms used by Trichoderma spp. in order to reduce the populations of other organisms, obtain space and nutrients, colonize the rhizosphere and achieve reproduction (Vinal et al., 2008; Hermosa et al., 2013). Moreover, when the Trichoderma spp. becomes established in the rhizosphere of its hosts, it promotes their growth with the production of indole acetic acid (Contreras et al., 2009; Zhang et al., 2013), organic acids (De Santiago et al., 2011; Hermosa et al., 2012) and siderophores (Aguado et al., 2012), which altogether favor nutrient assimilation and plant growth (Martínez et al., 2011; Samolski et al., 2012).

The efficiency of Trichoderma spp. to regulate plant-parasitic nematodes is mainly dependent on the fungal species, its place of origin, interaction with its plant host, and its adaptation to the environment where it will be applied (Mukhtar et al., 2013; Zhang et al., 2014). It has been reported that the reproduction and interaction with other microorganisms is favored by the application of Trichoderma strains, as they are already adapted to the environmental conditions from where they were taken (Affokpon et al., 2011; Candelero et al., 2015).

This study focused on exploring strains of Trichoderma spp. in the southeast of Mexico for their antagonistic effects against Meloidogyne spp. in the region. Previously, Candelero et al. (2015) reported that some strains have nematicidal activities in vitro and can enhance seedling growth in chili pepper. However, the effect of these Trichoderma strains on Meloidogyne spp. in chili pepper in vivo has not been tested. Therefore, the objective of this study was to estimate the ability of three strains of Trichoderma spp. to suppress reproduction of M. incognita and reduce the galling index of this nematode, and promote plant growth under greenhouse conditions.

MATERIALS AND METHODS

Origin of Trichoderma species. The Trichoderma species were obtained from the soil of southeast of Mexico, which have had no agricultural activity for over 30 years, using the unwashed particles technique of Bills et al. (2007) for their isolation, after which they were preserved in mineral oil and potato dextrose agar culture medium (DIBICO® S.A. de C.V. Mexico). Based on the National Biotechnology Information, the Trichoderma species were molecularly identified as T. harzianum-C1 (KJ028794), T. atroviride (HMO47766), T. virens (KF144629) and T. harzianum-C2 (KF201995). These species were selected as they present antagonistic activity in vitro against M. incognita (Candelero et al., 2015).

Preparation of Capsicum chinense seedlings for bioassay. Trichoderma species were activated in a potato dextrose agar culture medium and a concentration of spores was prepared, 1 x 10⁶ spores/mL according to Cubillos et al. (2009). C. chinense cv. Calakmul seeds were disinfected with 1% sodium hypochlorite for two minutes, followed by two washes with distilled water and planted in trays containing Sunrise™ commercial sterile substrate (Sun Gro Horticulture Canada Ltd.). A total of four inoculations with 1 x 10⁶ spores/mL concentrations were applied: in seed coats before planting and in the seedlings roots to drench at 10, 20 and 30 days after germination. Irrigations at field capacity were performed in accordance with the water needs of the crop, plus fertilization with Polyfeed® (17-17-17, Haifa, Mexico) twice a week, along with the irrigation water, for 47 days after sowing.

Obtaining J2 from M. incognita. Commercial plantations of C. chinense cv. Calakmul, naturally parasitized by M. incognita, were sampled in southeast of Mexico. Parasitized roots were placed in paper bags and stored in a refrigerator at 6 °C for 24 h. The samples were then washed with tap water and dissected with syringes under stereoscopic microscope Leica. The eggs were rinsed with sterile water and placed in sodium hypochlorite at 0.5% for two to three minutes, after which they were incubated at 25 ± 1 °C for three days until hatching of the J2 (Herrera et al., 2014). These were then concentrated in a flask of 500 mL to calibrate the inoculum. Perineal cuts of females removed from the galling roots, perineal patterns and taxonomic features of the nematode population under study were used to identify the specie (Einsenback & Triantaphyl- lou, 1991).
Trichoderma is antagonistic to M. incognita

**Management bioassay of M. incognita in C. chinense with Trichoderma spp. in the greenhouse.** In order to estimate the antagonism exerted by *Trichoderma* spp. on *M. incognita*, the substrate (soil) was steam sterilized (90 °C for three consecutive days). Plastic pots (two kg capacity) were filled with the sterilized substrate. Previously to transplanting, a hole of three cm diameter and five cm deep was prepared and inoculated with 1 mL of water containing 1000 eggs and larvae, and 300 J, of *M. incognita*; subsequently a 47 day old *C. chinense* plant, previously inoculated with *Trichoderma* spp., was transplanted in the pot (Herrera et al., 2014).

A total of six treatments were established: plants inoculated with I) *Trichoderma harzianum*–C1, II) *T. atroviride*, III) *T. virens*, IV) *T. harzianum–C2*, and V) SL. 24% oxamyl nematicida (Vydate®, Dupont, México, S. A. de C.V.) in doses of 1 mL/L of water, applied to the soil at the time of transplantation with the established inoculums; VI) Positive control (only with the nematodes). Fertilization with a 2:1:1 chemical equilibrium was considered for the nutritional management of the plants, with the sources: potassium nitrate (Ultrasol®, 12-00-46, SQM, México), monoammonium phosphate (MAP®, 12-61-00, Greenhow S.A. de C. V, Mexico) and urea (Magro®, 46-00-00, Fertinova, Mexico), applied twice a week for a period of 60 days. The treatments were kept at the greenhouse conditions of 28 ± 2 °C, with a relative humidity of 64% and lighting intensity of 450 luxes.

After 60 days the plants were harvested. Nematode damage intensity was measured by number of galls per root and galling index on the 0 to 6 scale classes according to Taylor & Sasser (1983), where 0 = complete and healthy root system, no infection, 1 = very few small galls only 1 to 10% of root system present, 2 = 11–25% of root system severely galled, 3 = 26 to 50% of root system severely galled, 4 = 51 to 75% of root system severely galled, 5 = 76–100% of root system severely galled, nearly no healthy roots). The number of eggs and number of females per gram of root were considered as nematode reproduction variables. To estimate these variables, total fragmentation of the root was conducted on each plant; this was then homogenized and 2 g of root were taken, 1 g was blended for 11 sec with sodium hypochlorite 2%; the eggs were counted in a compound microscope (4X, Leica), while the other gram was stained with acid fuchs in at boiling point for 10 minutes (Ayoub et al., 1980).

The stained roots were then deposited in vials with glycerin at 78% for subsequent dissection and counting of female adults, using a stereoscopic microscope (Leica M80). Growth estimators or plant agronomic vigor were also considered, such as plant height and root length (estimated with a measuring tape in cm), fresh root weight and dry shoot biomass (after drying at 65 °C for 72 h) in gram, and root volume by volumetric displacement in cm.

**Experimental design and statistical analysis.** Each treatment consisted of 10 plants (experimental unit), distributed under homogenous conditions in a completely randomized experimental design. A one way analysis of variance (ANOVA) was performed and in the case of the data relating to gallling index, which did not meet the assumptions of normal distribution and variance homogeneity, these were transformed by means of the arcsine function \[y = \arcsin\left(\sqrt{x/100}\right)\]. Tukey’s post hoc test (P<0.05) was used to identify which means significantly differ from each other, using the Statistical Analysis System version 9.3 of SAS Institute Inc.

**RESULTS**

Significant differences were obtained in the estimator variables (P<0.001), damage intensity and nematode reproduction. The number of galls per root with regard to the control was significantly diminished by the treatments with *Trichoderma* species and oxamyl (Table 1). The number of galls per root diminished by 68.18%, 67.73%, 66.78% and 65.07% with the treatments of *T. atroviride, T. harzianum–C1, T. harzianum–C2* and oxamyl, respectively, with regard to the control. However, a higher number of galls per root were estimated with *T. virens*, making it statistically lower than the other *Trichoderma* species and oxamyl. In the case of gallling index, the estimated values ranged from 6.75% to 39.50%; severity showed a significant reduction of 82.91% with *Trichoderma* spp. treatments while with oxamyl, a reduction of 70.25% was obtained, in comparison with the control.

The effect deriving from either *Trichoderma* species or oxamyl was evidenced by a significantly lower number of eggs per gram of root, with a reduction of 82.69% and 77.16%, respectively, with regard to the control (Table 1). The same effect prevailed in the case of the number of females per gram of root with the application of *Trichoderma* spp. and oxamyl, with reductions of up to 50%, with regard to the control. The application of *Trichoderma* species and oxamyl represented the same control of *M. incognita* in *C. chinense* for these estimator variables of nematode growth.

Higher growth records or plant agronomic vigor were the variables in which significant differences were estimated (P <0.001), due to the fact that they were generally favored by the suppressive effect of the *Trichoderma* species. Plant heights higher than those of the control were recorded with *Trichoderma* species and oxamyl application (Table 2). Control of the nematode, caused by oxamyl, favored the plant height 44.90 cm on average. A lower plant height average was registered with *T. virens* (39.60 cm); however, no statistical differences were revealed with the other *Trichoderma* species, where plant heights with averages ranging from 40.80 to 43.75 cm were estimated.

The treatments conformed by *Trichoderma* species and oxamyl were statistically different from the control, with respect to dry shoot biomass, fresh root weight and root volume. A higher dry shoot biomass production (9.82 to 10.69 g), roots with a higher fresh weight (47.32 to 54.37 g) and a higher root volume (52.4 to 57.70 cm<sup>3</sup>), in comparison with the control plants, were favored with the application of *Trichoderma* species in *C. chinense* (Table 2).
Table 1. Galling index and reproduction variables of *M. incognita* estimated in *C. chinense* at 60 days after planting, in the different treatments.

| Treatments               | Number of galls per root | Galling index (%) | Number of eggs per g root | Number of females per g root |
|--------------------------|--------------------------|-------------------|---------------------------|----------------------------|
| *T. harzianum*-C1        | 84.90 ± 7.86 c           | 6.75 b            | 149.20 ± 7.85 b           | 30.40 ± 4.64 b              |
| *T. atroviride*          | 83.70 ± 8.08 c           | 6.75 b            | 161.60 ± 15.33 b          | 28.80 ± 6.84 b              |
| *T. virens*              | 118.50 ± 6.27 b          | 10.50 b           | 153.41 ± 41.24 b          | 27.60 ± 4.35 b              |
| *T. harzianum*-C2        | 87.40 ± 14.39 c          | 6.75 b            | 132.90 ± 13.55 b          | 29.20 ± 1.75 b              |
| Oxamyl nematicide        | 91.90 ± 5.98 c           | 11.75 b           | 175.40 ± 13.81 b          | 25.70 ± 2.35 b              |
| Control                  | 263.1 ± 31.23 a          | 39.50 a           | 768.10 ± 67.74 a          | 59.20 ± 5.20 a              |
| SMD                      | 20.07                    | 8.40              | 45                        | 5.98                       |

The table shows average ± standard deviation. N=10. SMD=significant minimal difference. Same letters within the same column are statistically equal (Tukey, P<0.05). Oxamyl nematicide 24% applied at doses of 1 mL/L of water.

La tabla muestra promedios ± desviación estándar. N=10. SMD=Diferencia mínima significativa. Valores con las mismas letras dentro de cada columna son estadísticamente iguales (Tukey, P<0.05). Nematicida oxamyl 24% aplicado a dosis de 1 mL/L de agua.

Table 2. Growth variables estimated in *C. chinense* inoculated with *M. incognita* at 60 days after planting in the different treatments.

| Treatments               | Plant height (cm) | Dry shoot biomass (g) | Fresh Root weight (g) | Root Volume (cm³) |
|--------------------------|-------------------|-----------------------|-----------------------|-------------------|
| *T. harzianum*-C1        | 41.90 ± 4.97 ab   | 9.82 ± 1.41 a         | 51.03 ± 10.13 a       | 54 ± 6.99 a       |
| *T. atroviride*          | 40.80 ± 3.93 ab   | 10.61 ± 0.95 a        | 50.76 ± 5.00 a        | 54 ± 5.67 a       |
| *T. virens*              | 39.60 ± 3.62 b    | 9.94 ± 1.41 a         | 50.71 ± 4.12 a        | 52 ± 4.83 a       |
| *T. harzianum*-C2        | 43.75 ± 2.74 ab   | 10.42 ± 1.25 a        | 47.32 ± 4.72 a        | 53 ± 4.21 a       |
| Oxamyl nematicide        | 44.90 ± 3.14 a    | 10.69 ± 0.99 a        | 54.37 ± 4.59 a        | 57.70 ± 7.08 a    |
| Control                  | 29.10 ± 3.24 c    | 6.87 ± 1.10 b         | 30.82 ± 3.32 b        | 30.80 ± 3.29 b    |
| SMD                      | 4.86              | 1.59                  | 7.61                  | 7.3               |

The table shows average ± standard deviation. N=10. SMD=significant minimal difference. Same letters within the same column are statistically equal (Tukey, P<0.05). Oxamyl nematicide 24% applied at doses of 1 mL/L of water.

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**DISCUSSION**

The *Trichoderma* species evaluated were capable of inducing an antagonistic effect *in vivo* against *M. incognita* with nematode control effects equal to the nematicide oxamyl. The antagonistic effect of these species against the nematode persisted in the study conditions evaluated; such results were actually expected as their antagonistic activity against the *J* stage of *M. incognita* has been shown *in vitro* in previous reports by Candelero et al. (2015).

The potential biocontrol of *Trichoderma* spp against nematodes has also been demonstrated with *T. harzianum* against *M. incognita* (Sahebani & Hadavi, 2008; Naserinasab et al., 2011; Moura et al., 2012) and *M. javanica* (Sharon et al., 2001; Feyisa et al., 2015) with *T. atroviride* against *Meloidogyne* spp. (Mendoza et al., 2013) and *T. virens* against *M. incognita* (Meyer et al., 2001). As revealed by the results obtained herein, these species have significant potential as biological control agents against *M. incognita*; furthermore, due to their control ability in diverse conditions, they are able to bond together for the integrated management of the diseases caused by *Meloidogyne* spp. (Sariah et al., 2005; Guédez et al., 2008).

In this study, direct effects on nematode reproduction, caused by *Trichoderma* species, were observed, with reductions in egg production and formation of females. As a consequence, fewer galls were formed, with regard to the control. The largest number of galls, compared with the other *Trichoderma* species, were presented by *T. virens*. However, this did not represent a lower nematode control, since the rest of the galls in the roots inoculated with *T. virens* were smaller in size.
in comparison with the other treatments. This particular ability to limit nematode reproduction when *Trichoderma* species were inoculated was also reported by Al-Hazmi & Tariq B. (2016), in *Solanum lycopersicum* plants when *T. harzianum* and *T. viride* against *M. javanica* decreased (1) egg formation by 44% and 63%, respectively, and (2) root gall formation by 79% and 88% respectively; the application of *T. virens* against *Meloidogyne* spp. in *Cucumis sativus* decreased gall formation by 50% (Zhang et al., 2014).

The control variations caused by *Trichoderma* spp. against nematodes have been estimated in other study models, such as *C. annuum-M. incognita* (Meyer et al., 2000), *C. sativus-M. incognita* (Moura et al., 2012) and *Cucurbita pepo-M. javanica* (Sokhandani et al., 2016). This suggests that the ability of *Trichoderma* spp. to suppress *Meloidogyne* spp. reproduction could be a result of the aggressiveness of each fungal species, which is influenced by the origin and interaction of biotic and abiotic factors (Moosavi & Zare, 2015). Hence, as biological control agents or any other management technique is incorporated, they must have the ability to suppress the nematode population so that the amount of eggs, females and galls become determining factors for the handling of phytopathogenic species (Al-Hazmi & Tariq B. (2016). In the case of microorganisms, these have a greater effect as the roots of their hosts are colonized (Meyer et al., 2000; Meyer et al., 2001) which can occur quite frequently with the application of arbuscular mycorrhizal fungi capable of reducing the reproduction of *M. incognita*, *M. javanica* and *Rotylenchulus reniformis* (Cristóbal et al., 2010; Lax et al., 2011; Herrera Parra et al., 2014).

The biocontrol effect of *Trichoderma* spp. is related to antibiosis (Ramírez, 2014; Sokhandani et al., 2016) where lytic enzymes, such as glucanases and proteases determine egg infeasibility, and the reduction of nematode penetration (Sahebani et al., 2008; Szabó et al., 2013). There is another action mechanism associated with the activation of plant defense mechanisms when the rhizosphere is colonized by *Trichoderma* spp. establishing an endophyte interaction with the host (Hermosa et al., 2012; Zhang et al., 2014).

The application of strains *Trichoderma* spp., studied as *M. incognita* antagonistic, did not promote *C. chinense* growth, since the same effect on estimated agronomic variables was recorded with the nematicide oxamyl treatment. Such growth promotion takes place when a single interaction with *Trichoderma* spp. is established by the host and indole acetic acid is produced by this fungi, which is associated with plant biomass production (Contreras et al., 2009; Martínez et al., 2011). This does not happen, however, when an interaction with a pathogen is established by the host; instead, the biocontrol microorganisms, such as *Trichoderma*, make resources from the plant intended for the activation of defense mechanisms, known as induced systemic resistance, through the pathways of jasmonic acid, salicylic acid or ethylene (Hermosa et al., 2013; Nawrocka et al., 2013).

The efficiency and potential of the application of the *Trichoderma* species evaluated herein, as an alternative to the use of nematicides, is evident from the results obtained in this study, where the effect of their application was found to be equivalent to that of the nematicide oxamyl, with the advantage that these microorganisms do not select populations of resistant nematodes or cause environmental pollution (Bale et al., 2008; Hernández et al., 2011; Xie et al., 2015).

### CONCLUSIONS

The fungal species studied in this work have potential as antagonistic agents against *M. incognita*, given that they present the same capacity as the nematicide oxamyl to reduce the severity and reproduction of the nematode and they did not promote the growth of plants. Their application as antagonists becomes potentiated when applied within the kind of environments to which they are already adapted and also when there is compatibility with the crop of interest. Further studies should consider the persistence of *Trichoderma* spp. antagonistic activity against *M. incognita* when it is incorporated into several *C. chinense* production cycles.

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