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Controle biológico de *Meloidogyne incognita* (Kofoid & White) Chitwood, mediante a aplicação de agentes de controle biológico

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Abstract

The objective of the research was to determine the potential of *Trichoderma* spp. strains, *Isaria fumosorosea*, and *Isaria javanica* as biocontrol agents against *Meloidogyne incognita* (Kofoid and White) Chitwood, obtained from tomato cv. Saladette (*Solanum lycopersicum* L.). Strains of *T. harzianum*, *T. viride*, *T. koningii*, *T. asperellum*, and a *Trichoderma* sp. isolate, as well as *I. fumosorosea* and *I. javanica*, were used. These were previously selected for their high parasitic capacity, antibiosis, and adaptation to diverse environmental conditions and substrates. In the *in vitro* assays, parasitism of the biological control agents (a pure filtrate of each strain) on eggs, oothecae, and juveniles (J2) of *M. incognita* was evaluated. Observations were carried out using an optical microscope with a 40X objective lens at 10 days and 72 hours, respectively. Nematodo control under semi-controlled conditions was conducted in an experimental area using 10 kg polyethylene bags, inoculated with approximately 5,000 juveniles (J2) and planted with tomato seedlings. Seven days after nematode inoculation, the biological agents were applied to the soil; 45 days later, incidence and severity variables were evaluated. Based on the results obtained, it was found that the strains of *T. harzianum*, *T. asperellum*, *T. koningii*, and *I. fumosorosea* are efficient for the control of different stages of the biological cycle of *M. incognita*.

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Resumen

El objetivo de la investigación fue determinar el potencial de cepas de *Trichoderma* spp*., Isaria fumosorosea* e *Isaria javanica* como agentes de biocontrol sobre *Meloidogyne incognita* (Kofoid y White) Chitwood, procedente de jitomate c.v. Saladette (*Solanum lycopersicum* L.). Se utilizaron cepas de *T. harzianum, T. viride, T. koningii, T. asperellum* y un aislado de *Trichoderma* sp., *I. fumosorosea* e *I. javanica*, seleccionadas previamente por su alta capacidad parasítica, antibiosis y adaptación a diversas condiciones ambientales y sustratos. En los ensayos *in vitro* se evaluó el parasitismo de los agentes de control biológico (un filtrado puro de cada una de las cepas) sobre huevos, ootecas y juveniles (J-2) de *M. incognita*. Las evaluaciones se realizaron con un microscopio óptico con objetivo 40X a los 10 días y 72 horas respectivamente. El control del nematodo en condiciones semicontroladas se llevó a cabo en el área experimental utilizando bolsas de polietileno con capacidad de 10 kg, inoculadas con aproximadamente 5.000 juveniles (J2) y sembradas con plántulas de jitomate. A los siete días posteriores a la inoculación del nematodo, se aplicaron los agentes biológicos al suelo; 45 días después se evaluaron las variables de incidencia y severidad. Con base en los resultados obtenidos, se obtuvo que las cepas de *T. harzianum, T. asperellum, T. koningii* e *I. fumosorosea* fueron eficientes para el control de *M. incognita* en diferentes etapas del ciclo biológico.

Palabras clave: jitomate, parasitismo, *Trichoderma* spp*., Isaria fumosorosea, Isaria javanica.*

Resumo

O objetivo da pesquisa foi determinar o potencial de cepas de *Trichoderma* spp., *Isaria fumosorosea* e *Isaria javanica* como agentes de biocontrole sobre *Meloidogyne incognita* (Kofoid e White) Chitwood, proveniente de tomate cv. Saladette (*Solanum lycopersicum* L.). Foram utilizadas cepas de *T. harzianum*, *T. viride*, *T. koningii*, *T. asperellum* e um isolado de *Trichoderma* sp., *I. fumosorosea* e *I. javanica*, previamente selecionadas por sua alta capacidade parasítica, antibiose e adaptação a diversas condições ambientais e substratos. Nos ensaios *in vitro*, foi avaliado o parasitismo dos agentes de controle biológico (um filtrado puro de cada uma das cepas) sobre ovos, massas de ovos e juvenis (J2) de *M. incognita*. As avaliações foram realizadas com microscópio óptico com objetiva de 40X, aos 10 dias e 72 horas, respectivamente. O controle do nematoide em condições semicontruladas foi realizado na área experimental utilizando sacos de polietileno com capacidade de 10 kg, inoculados com aproximadamente 5.000 juvenis (J2) e transplantados com mudas de tomate. Sete dias após a inoculação do nematoide, os agentes biológicos foram aplicados ao solo; 45 dias depois foram avaliadas as variáveis de incidência e severidade. Com base nos resultados obtidos, verificou-se que as cepas de *T. harzianum*, *T. asperellum*, *T. koningii* e *I. fumosorosea* foram eficientes no controle de *M. incognita* em diferentes estágios do seu ciclo biológico.

Palavras-chave: tomate, parasitismo, *Trichoderma* spp., *Isaria fumosorosea*, *Isaria javanica*.

Introduction

The tomato (*Solanum lycopersicum* L.) is one of the most widely consumed vegetables worldwide and has significant economic value; it ranks eleventh among the most widely produced crops globally. Demand for tomatoes is increasing year on year, which has driven their cultivation, production and marketing. In Mexico, 41,479.77 ha were planted in 2022, yielding 2.9 million tonnes with an estimated economic value of 14.759 billion pesos (Agri-Food and Fisheries Information System [SIAP], 2024). The tomato exhibits a remarkable ability to adapt to diverse climatic conditions and soil types, which facilitates its establishment in different regions of the country. However, in tropical countries, tomato cultivation is characterised by a high incidence of plant-parasitic nematodes, which represent a global threat to agricultural productivity (Sikora *et al*., 2018).

More than 4,100 species of plant-parasitic nematodes have been documented, including cyst nematodes (*Heterodera* spp. and *Globodera* spp.), lesion nematodes (*Pratylenchus* spp.) and root-knot nematodes (*Meloidogyne* spp.) (Nicol *et al*., 2011). In particular, *Meloidogyne* species cause losses of between 20 and 33 %, affecting more than 90 % of economically important crops, in both traditional and protected production systems (Ayaz *et al*., 2024; Ning *et al*., 2022). These nematodes invade the root system, disrupting the uptake of water and nutrients, which significantly reduces crop growth and yield (Migunova and Sasanelli, 2021). Furthermore, they weaken the plants' defences, making them more susceptible to secondary pathogens, and secrete effector proteins that disrupt the host's defence mechanisms (Ali *et al*., 2023).

The control of plant-parasitic nematodes has traditionally relied on the use of chemical nematicides, due to their proven effectiveness. However, in many countries their use has been restricted or even banned, due to their negative effects on the environment, human health and the depletion of the ozone layer. For this reason, it is necessary to develop innovative control alternatives with low environmental impact, such as biological nematicides, which are low-cost, environmentally friendly and less harmful to the host. In this context, evaluations have been conducted on microorganisms that act directly or indirectly against nematodes through competition for nutrients and niches, characterised by the production of lytic enzymes, antibiotics and volatile toxic metabolites (Ayaz *et al*., 2024; Migunova and Sasanelli, 2021).

Based on the above, the aim of this study was to evaluate the potential of strains of *Trichoderma*, *Isaria fumosorosea* and *Isaria javanica* as biocontrol agents against *Meloidogyne incognita* (Kofoid and White) Chitwood, isolated from tomato crops c.v. Saladette (*Solanum lycopersicum* L.), through *in vitro* trials and under semi-controlled conditions.

Materials and methods

Isolation of nematodes

Nematodes were isolated at the Microbiology Laboratory of the Southern Technological University in the state of Morelos, Puente de Ixtla municipality, Morelos, Mexico (18°36'51" N, 99°19'15" W, 900 m a.s.l.), from tomato plants (c.v. Saladette) exhibiting typical symptoms of the disease and collected at random from different locations in Morelos, Mexico. The samples collected in the field

were transported to the laboratory in polyethylene bags lined with moistened Kraft paper to prevent desiccation and were stored at 16 °C until processing. The galls were washed with plenty of drinking water and disinfected with 1 % sodium hypochlorite for 20 s; the samples were then washed three times with sterile distilled water until the sodium hypochlorite was removed. The oothecae were obtained directly from the gall tissues, which were finely cut with a scalpel. The eggs were obtained by blending fragments (15) of diseased root tissue, 1–2 cm in length, for 30 s in a 0.5 % sodium hypochlorite solution diluted 1:10 with distilled water. Subsequently, the homogenised tissue was passed through 200 and 500 mesh sieves, with the contents of the second sieve collected in a 500 mL beaker and counted on a counting plate until a concentration of 100 eggs. mL^{-1} was reached (Vrain, 1977).

The eggs and second-instar (J2) larvae of *M. incognita* were obtained from the roots of infected plants using the maceration and filtration method (Hooper *et al*., 2005). To do this, 25 g of roots were placed in 200 mL of water and blended for 30 seconds. The suspension was decanted through 200- and 325-mesh sieves and then transferred to a separating funnel. After 48 h of standing, 20 mL of the solution was extracted; from this volume, 3 mL was analysed on a watch glass. The presence of J2 was quantified (five counts) under an optical microscope with a 40X objective (LABOMED, Inc., USA).

In vitro* parasitic effect of biological agents on *Meloidogyne incognita

For the mycoparasitism trials, biological agents from the strain collection of the Southern Technological University in the state of Morelos, Puente de Ixtla municipality, Morelos, Mexico, were used. The fungi were cultured (two passages) in Petri dishes (90 mm) containing potato dextrose agar (PDA; BD Bioxon) for five days for *Trichoderma* spp. and seven days for *I. javanica* and *I. fumosorosea*, respectively. The dishes were sealed with Parafilm® and incubated at 26 °C. From the colonies developed previously, conidial suspensions of each agent to be evaluated were prepared under aseptic conditions in a laminar flow cabinet (Biobase, BKCB-H1500, China). To do this, 10 mL of sterile distilled water was added to each individual colony, and the mycelium was detached using a Drigalski spatula to obtain a conidial suspension, which was homogenised at 1,800 rpm for 60 s in a vortex mixer (IKA Vortex 2, Germany). The concentration was adjusted to 10^7 $CFU.mL^{-1}$ using a Neubauer chamber (Marienfeld, Germany).

To assess the effect of different species of *Trichoderma* and *Isaria* on nematode eggs and oothecae, 96 well microtitre plates were used. The experiments were set up using a completely randomised design, with seven treatments corresponding to the different microorganisms evaluated and one control treatment. Each treatment had four replicates, with each well considered as an experimental unit. To each well, 200 µL of a conidial suspension adjusted to a concentration of 1×10^7 $CFU.mL^{-1}$ of the microorganism under evaluation was added (Siddiqui and Mahmood, 1999). The control treatment consisted of the addition of 200 µL of sterile distilled water. Subsequently, five oothecae and 20 eggs per well were added to each treatment (Hussey and Barker, 1973). The plates were sealed with Parafilm and incubated at 26 °C for 10 d. Once the incubation period was complete, the effect of the treatments on the eggs and oothecae was assessed. The structures were collected separately and placed on microscope slides for observation of parasitism (Sharon *et al*., 2001), using an optical microscope (40X).

To determine the effect of biological agents on the J2 larval stage of *M. incognita*, 10 larvae were placed in each well, with four replicates per treatment (Hussey and Barker, 1973). Subsequently, 200 µL of a conidial suspension of each strain, adjusted to a concentration of 1×10^7 $CFU.mL^{-1}$, was added. After 72 h of exposure, the juveniles from each treatment were removed and transferred to a new plate containing 200 µL of sterile distilled water for 48 h. Dead juveniles were collected and mounted on slides for observation under an optical microscope (40X) to verify the presence of parasitism (Sharon *et al*., 2001). In both trials, visual evidence was obtained using a digital camera (20 MP) (Canon® PowerShot ELPH 180 8X, Japan).

To determine the most effective agents for nematode control, the data were transformed by $\sqrt{x+1}$. Means were compared using Fisher's least significant difference (LSD) test, with a significance level ($p\leq0.05$), using the InfoStat Professional version 2.1 statistical package (Di-Rienzo *et al*., 2017).

In order to validate their efficacy under semi-controlled or greenhouse conditions, the strains evaluated *in vitro* that demonstrated the greatest parasitism capacity against *Meloidogyne incognita* were selected.

Effect of selected biological agents on *M. incognita* under semi-controlled conditions

The experiment was carried out in a shade house at the Southern Technological University in the state of Morelos, Puente de Ixtla, Morelos, Mexico. During the vegetative phase of the tomato crop (cv. Saladette), average temperatures of 25 °C during the day and 17 °C at night were recorded, with relative humidity of 65 %; these climatic conditions were suitable both for the crop and for the activity of the biological agents under evaluation (Lewis and Papavizas, 1983).

The seedlings were grown in polystyrene trays (lightweight, insulating containers made of expanded polystyrene (EPS) with 200 cells, commonly used in agriculture for germinating seeds). Two seeds were sown per cell, which was covered with a 1 cm layer of growing medium consisting of Sphagnum peat moss and organic matter in a 3:1 (w:w) ratio, previously sterilised in an autoclave. The trays were placed in the experimental area and watered every third day until the seedlings emerged. After emergence (15 days), when the seedlings reached 15 cm in height, they were transplanted into 10 kg polyethylene bags (30 × 32 cm), containing a mixture of soil, peat moss and organic matter (cow manure) in a 2:1:1 ratio. The substrate was first sterilised in an autoclave (120 °C for 30 m, followed by two consecutive cycles with a 24 h interval) and subsequently disinfected with potassium soap. Additionally, the soil was covered with a dark cloth to aid the disinfection process. Watering was carried out every other day.

Seven days after transplanting, the pots were inoculated with 2,500 ± 10 second-stage (J2) juveniles of *M. incognita*, equivalent to 2.5 J2 per gram of soil (Sikora *et al*., 2018). Seven days after nematode inoculation, conidial suspensions of the selected biological agents, adjusted to a concentration of 1×10^7 $conidia.mL^{-1}$, were applied at a rate of 100 mL per bag. Prior to application, three drops of Tween 20 were added to each suspension to improve inoculum dispersion.

The strains used in each treatment were previously cultured in 90 mm diameter Petri dishes containing potato dextrose agar (PDA; BD Bioxon) and incubated at 26 °C for 10 days until full colony growth and spore maturation were achieved. Conidial suspensions were prepared in a laminar flow cabinet from the developed colonies; to do this, 10 mL of sterile distilled water was added to each colony and the mycelium was scraped off using a metal spatula. Subsequently, the

suspensions were homogenised using a vortex mixer and quantified using a Neubauer chamber.

The experiment was set up using a completely randomised design, with eight replicates (bags) per treatment, resulting in a total of 56 plants in the experimental area. The treatments evaluated were: 1) *Trichoderma harzianum*; 2) *Trichoderma koningii*; 3) *Isaria fumosorosea*; 4) *Trichoderma* sp.; 5) *Trichoderma asperellum*; 6) T-combination (consortium of *T. koningii* + *T. harzianum* + *T. asperellum* + *I. fumosorosea*); and 7) absolute control, consisting of tomato plants (cv. Saladette) inoculated solely with *M. incognita* and without the application of biological agents. Incidence and severity were determined 45 days after transplanting.

Incidence

The number of plants affected by the nematode was determined according to the formula;

$$\text{Incidence} = \frac{\text{Number o affeted plants}}{\text{Total plants}} \text{ X } 100$$

Severity was determined by assessing the galls index (GI). To do this, the Saladette cultivar tomato plants were removed from the bags without damaging the root system. The roots were then washed, and the percentage of *Meloidogyne* infection (root surface affected by galls) was assessed macroscopically, using the severity scale proposed by Taylor and Sasser (1978), as shown in figure 1.

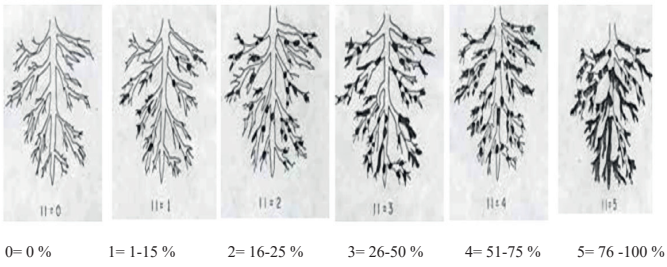

Figure 1. Severity scale according to Taylor and Sasser (1978). Percentage of root surface area affected by galls caused by *M. incognita*.

To determine the most effective microorganisms for controlling the nematodes, the incidence data were transformed using the formula √x+1. A one-way analysis of variance (ANOVA) was performed and the means were compared using Fisher's least significant difference (LSD) test ($p \leq 0.05$) using the InfoStat Professional version 2.1 statistical package (Di-Rienzo *et al*., 2017).

Results and discussion

Identification of *Meloidogyne incognita*

Morphological characteristics of females and males

The isolated nematodes exhibited certain characteristics that allowed them to be identified as *M. incognita*. The females were hyaline, pear-shaped to rounded. The stylet was cone-shaped, curved towards the dorsal side, with the widest part at the base and broad, flat nodules. The males were filiform, with a robust stylet and two rings in the cephalic region; the anterior part of the stylet was 'paddle'-shaped with a blunt tip; the basal nodules were flat and rounded, with a slight separation from the body (Eisenback *et al*., 1981) (Figures 2A and B).

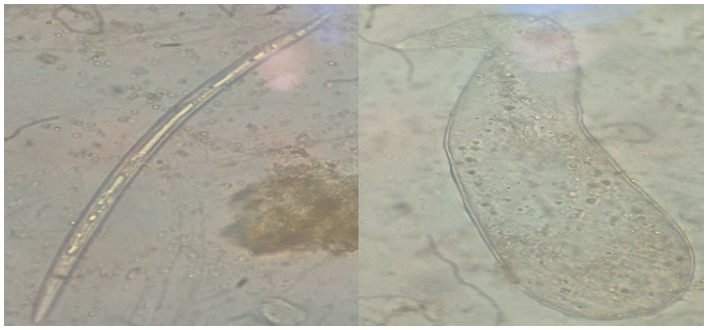

Figure 2. Morphological characteristics of adult *Meloidogyne incognita*. A) Males and B) Females.

The eggs appeared small, translucent to slightly yellowish and oval in shape (Figure 3A) when observed directly from the galls produced on the roots during the final stages of development (Calderón-Urrea *et al*., 2016). Oothecae were observed clustered in a mucilaginous matrix (Figure 3B) on the outside of the host plant's roots, in direct contact with the soil (Subedi *et al*., 2020). The presence of these oothecae on the roots is evidence of infection by *M. incognita* and can be used for the diagnosis of the disease.

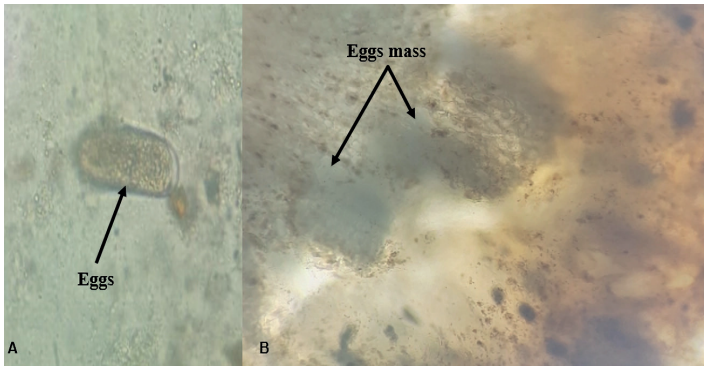

Figure 3. Eggs (A) and oothecae (B) of *Meloidogyne incognita*. Note the gelatinous mass covering the eggs (B).

The life cycle comprises four larval stages. The first stage develops inside the egg (Figure 4A). Of the remaining stages, only the second stage can be found in the soil; the third and fourth stages (like the female) are strict endoparasites of roots; these are visible in the samples (Figure 4B). These characteristics resemble those described by Martínez-Gallardo *et al*. (2019) for the species *M. incognita*.

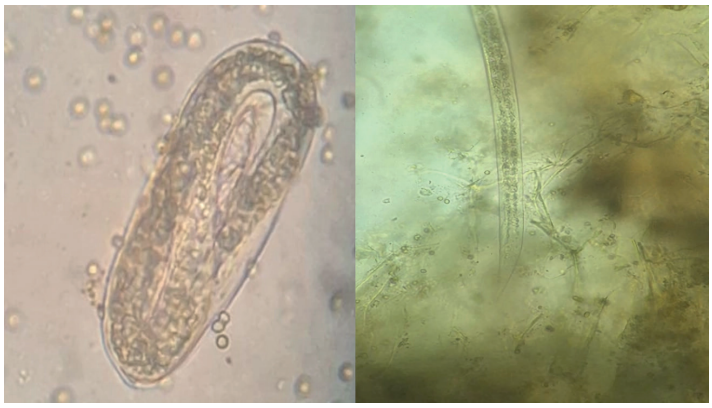

Figure 4. Larval stages of *Meloidogyne incognita*. A) Juvenile in an unhatched egg; B) Hatched juvenile.

In vitro* parasitic effect of biological agents on *Meloidogyne incognita

In general, microscopic examination revealed that all strains exhibited parasitic activity against the various stages of the nematode (Figure 5A, B and C).

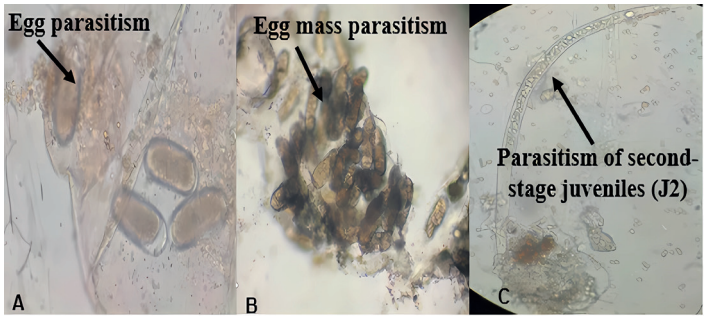

Figure 5. Parasitism of *Trichoderma against* the different life stages of *Meloidogyne incognita*. A) Parasitism on eggs, B) Parasitism on oothecae and C) Parasitism of infective J2 juveniles.

Regarding the eggs of *M. incognita*, all strains and the *Trichoderma* isolate exhibited high parasitic activity (Table 1), except for *T. asperellum* and *I. javanica*.

Table 1. *In vitro* parasitism of *Trichoderma* spp. and *Isaria* spp. strains on *Meloidogyne incognita* isolated from tomato plants plants c.v. Saladette (*Solanum lycopersicum* L.) Amacuzac municipality, Morelos, Mexico.

Treatment	Eggs	Oothecs	J2 Adult
T. koningii	9.80^{a}	9.79^{a}	9.26^{a}
T. harzianum	9.66^{a}	9.79^{a}	1.00^{c}
T. sp	9.53^{a}	8.97^{ab}	2.79^{c}
T. viride	8.85^{ab}	9.53^{ab}	6.30^{b}
I. fumosorosea	8.25^{b}	8.32^{ab}	9.23^{a}
T. asperellum	1.00^{c}	7.76^{b}	9.23^{a}
I. javanica	1.00^{c}	1.00^{c}	1.00^{c}
Control	1.00^{c}	1.00^{c}	1.00^{c}
CV	6.57	11.10	19.97
DMS	1.03732	2.01042	2.54438

Means with the same letter in the same column are not significantly different according to Fisher's LSD test ($p \leq 0.01$). T: *Trichoderma*, I: *Isaria*, CV: coefficient of variation, LSD: least significant difference. MSD: minimum significant difference.

These strains caused disruption to the internal contents of the eggs and exhibited mycelial growth and spores (*T. harzianum*) within them, an event indicating that the fungus had penetrated the egg (secretion of extracellular enzymes by the fungi) and that the embryos had died. Regarding the oothecae, the strains *T. koningii* and *T. harzianum* showed superior results to *T. asperellum* and *I. javanica*, with no statistical differences compared to the treatments *T. viride*, *I. fumosorosea* and *Trichoderma* sp. Meanwhile, regarding J2 juveniles, the best results were obtained with *T. koningii*, *I. fumosorosea* and *T. asperellum*, with no differences between them, but with differences compared to the other treatments.

It has been reported that some species of the genus *Trichoderma* are capable of parasitising eggs and second-stage juveniles (J2) of root-knot nematodes (*Meloidogyne* spp.) (Druzhinina *et al*., 2011; Herrera-Parra *et al*., 2018; Mukhtar *et al*., 2021). Infection of the eggs by strains of *Trichoderma* spp. is possible due to increased activity of enzymes such as chitinases, proteases and lipases when the fungus comes into contact with the eggs or juveniles (Sahebani and Hadavi, 2008); this destroys the egg shell and allows penetration. Sharon *et al*. (2007) demonstrated that the gelatinous matrix in which the eggs are laid promotes the attraction of the fungus and enhances the parasitic capabilities of numerous *Trichoderma* isolates, which utilise this matrix as a nutrient source. Benedetti *et al*. (2021) reduced the number of eggs by 50 % through the use of *Trichoderma* spp. Al-Ani *et al*. (2022) and Blanco *et al*. (2024) demonstrated that *Paecilomyces lilacinus* is a facultative parasite of eggs from a wide range of plant-parasitic nematodes, as well as attacking cysts and adult females.

Based on the results obtained, *I. javanica* was not considered for subsequent experiments, due to the absence of a significant effect on the variables evaluated.

Effect of selected biological agents on *M. incognita* under semi-controlled conditions

Incidence

During the evaluation period, symptoms of the disease were observed, characterised by slight yellowing of the leaves, accompanied by reduced growth, as well as a subsequent delay in the flowering of the crop. At the time of sampling, the incidence ranged from 15 to 100 %, with T-combined, *T. harzianum*, *T. koningii* and *I. fumorosea* being the treatments with the lowest incidence, with no statistical differences between them. However, the control plants were found to be completely affected (Table 2).

Table 2. Effect of fungal strains on the incidence and severity of *M. incognita* in tomato seedlings of the c.v. 'Saladette' (*Solanum lycopersicum* L.), Amacuzac municipality, Morelos, Mexico.

Treatment	Incidence	Severity (%)	Grade
T. harzianum	2.16^{a}	1.49^{a}	1
T. koningii	3.05^{ab}	1.78^{a}	1
I. fumosorosea	3.05^{ab}	2.03^{ab}	1
T. sp	4.51^{b}	2.72^{b}	1
T. asperellum	6.38^{c}	4.04^{c}	2
T- combinado	1.80^{a}	1.00^{a}	0
Control	9.79^{d}	8.18^{d}	4
CV	23.33	16.50	-
DMS	1.67226	0.82676	-

Means with the same letter in the same column are not significantly different according to Fisher's LSD test ($p \leq 0.01$), CV: coefficient of variation; MSD: minimum significant difference.

Severity

Severity ratings for the different treatments ranged from 0 to 2, with the lowest severity ratings (grade 1) obtained with the *T.* combined treatment and the *T. harzianum* strain, showing no statistical differences compared with *T. koningii* and *I. fumosorosea*, in which the plant exhibited slight galls. However, *T.* sp. and *T. asperellum* showed differences compared to these three strains; although they did not exceed severity grade 1 either. All treatments showed differences compared to the control (Table 2).

Trichoderma species are widely distributed in soil and possess parasitic and antibiotic properties. Their metabolic capacity and their ability to compete for space and nutrients in the wild make them highly effective in agricultural applications (Harman, 2024). Through the combined use of *Trichoderma* strains selected *in vitro*, a minimal percentage of galls was observed on the plants. Kredics *et al*. (2024) note that the control effect is greater when consortia of microorganisms of the same or different species are applied, as they can broaden the range of pathogen control. Furthermore, Moo *et al*. (2018) demonstrated that mixtures of different species of the genus *Trichoderma* reduced the severity of *M. incognita* on the roots of *S.*

lycopersicum by more than 80 %, as well as decreasing the number of eggs and reducing the number of females by more than 90 %.

Conclusions

The results of the *in vitro* and semi-controlled field trials suggest that, in protected vegetable production, the combined use of the strains studied (*T. harzianum*, *T. asperellum*, *T. koningii* and *I. fumosorosea*) and of the individual strains of *T. harzianum*, *T. koningii* and *I. fumosorosea* is effective in controlling the different stages of the life cycle of *M. incognita*.

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