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Molecular Characterization and Pathogenicity of *Trichoderma* Isolates to *Meloidogyne javanica*

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Abstract

Nematodes are considered a serious problem for agriculture. Nematodes of the *Meloidogyne* genus can attack a wide range of plants, needing different management methods to decrease its population. Fungi from the *Trichoderma* genus has been related to have potential as biological control agents. However, before an organism is used as biological control agent, first it is necessary to prospect, characterize and test its potential as biocontrol agent, so the objective of this work was to characterize and test fungi isolates of the *Trichoderma* genus to control *M. javanica*. We obtained forty isolate to carry out this experiment. We extracted the DNA of each isolate to discover which species we were testing, by doing a PCR and sequencing. We tested *in vitro* their parasitism effect using ELISA plate. Also, we extracted their filtrate to see if their metabolites have potential to reduce nematode population by showing a high mortality or inhibiting hatching. The results confirmed the high potential of the fungi of *Trichoderma* genus as a biological agent to control *Meloidogyne javanica*.

Keywords: Biological control, Integrated management, root-knot nematode, fungal filtrates, nematicide

1. Introduction

Soybean (*Glycine max* L.) is one of the most important agricultural commodities in the world. For this reason, it is constantly sought to increase productivity without increasing the cultivated area. Yield can be affected by several factors, among them are diseases caused by plant-parasitic nematodes, an important cause of reduced grain production in this crop. Worldwide, there are approximately 100 species of known plant-parasitic nematodes that decrease the production of this commodity [1]. In Brazil, these species are distributed primarily among the genera *Meloidogyne*, *Heterodera*, *Pratylenchus* and *Rotylenchus* [1–4]. Among these species, the root-knot nematodes, specifically *Meloidogyne javanica*, stands out because it has the ability to parasitize and cause significant damage to soybeans [5, 6].

Agricultural systems have had little crop diversification over the years, which means that these organisms have good availability of food throughout the year.

This availability causes the nematode population to grow more and more, making control even more difficult. Therefore, new control alternatives are being studied to minimize the damage caused by these organisms. Currently, biological control within integrated management stands out as an efficient and economically viable alternative to the use of chemical nematicides [7, 8]. In general, biological products have low toxicity and environmental risk, and we would be using a wide variety of microorganisms that can naturally parasitize nematodes and their eggs in natural and agricultural environments. Among the main groups of microorganisms responsible for the biocontrol of nematodes, fungi stand out, representing up to 75% of the microorganisms used in the control of plant-parasitic nematodes [9, 10]. The ability to colonize the soil and persist for a long period makes these organisms increasingly visible. When we think about long persistence, we are comparing it with chemicals, which do not have a long-lasting residual. We can observe in the field chemical products that are used in seed treatment in soybean culture lose their efficacy even before the reproductive period of the culture.

Therefore, the possibility of incorporating organisms that have a long persistence in the soil can be a very important control measure, given the worrying scenario that the nematodes have been presenting. Fungi of the *Trichoderma* genus are considered one of the most used and promising in biological control [9]. This can be explained due to the versatility of the mechanisms of action against agricultural diseases and pests. *Trichoderma* species are capable of using different mechanisms of action, such as parasitism, production of metabolites (antifungal substances and antibiotics), production of polymer and protein degrading enzymes (glucanases, chitinases and proteases) [11]. In addition, these fungi can stimulate plant growth (production of phytohormones) and induce systemic resistance against diseases, which makes their use in agricultural systems even more promising [11].

Besides that, some isolates also have survival strategies that make them highly competitive in the environment, such as: survival in acid and / or saline soils; survival in conditions of high temperature and low humidity; fungicide resistance; adaptation to different environments and climatic zones, as they can inhabit soils in tropical regions and temperate climates; production of resistance structures; high efficiency in the use of resources as nutrients, thus making them excellent competing organisms; extraordinary capacity for proliferation in the rhizosphere and communication with plants, among others.

Due to the variation of environmental conditions (soil, climate, vegetation, etc.) on the planet, a species may have strains (variants) with specific adaptations to different environments. Thus, it is suggested that the antagonistic activity of a *Trichoderma* strain may change in relation to the same organism, if the fungus is used in regions with a microclimate different from the original fungus isolation site. This makes the search for antagonist agents from different regions relevant for obtaining isolates with potential application in biological control [12]. In this context, the objective of this work was to characterize and test fungi isolates of the *Trichoderma* genus to control *M. javanica*.

2. Material and methods

2.1 *Meloidogyne javanica* inoculum

The root-knot nematode inoculum, specifically *Meloidogyne javanica* (Est. J3), was obtained from a commercial soybean crop (*Glycine max*) in the municipality of Júlio de Castilhos, Rio Grande do Sul (29°04'55.5"S 53°41'07.7"W). Subsequently,

the nematodes were extracted from the soybean roots by the method that consists of grinding in a blender with the addition of 0.5% sodium hypochlorite followed by sifting and centrifugation with sucrose solution, carried out at the Soil Biology laboratory of the Federal University of Santa Maria (UFSM). The extracted nematodes were inoculated in tomato plants cultivar “Santa Cruz” (*Solanum lycopersicum* L.), to maintain the population. The tomatoes remained in a greenhouse with temperature controlled at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$. The females of the population were periodically submitted to electrophoresis with the esterase enzyme [13] to confirm the purity of the population.

2.2 In vitro evaluation of the nematicidal and nematostatic effect of *Trichoderma* spp. on *Meloidogyne javanica*

2.2.1 Obtaining *Trichoderma* isolates

Forty *Trichoderma* isolates from different regions of Brazil were used in this study showing the city that the isolates were provided (**Figure 1**). Isolates provided by private companies are from the Southeast and Midwest regions, however their locations were not provided.

Of the 40 isolates used in this study, 6 isolates are from the fungi bank of the laboratory of Soil Biology, 10 isolates were provided by the Federal University of Santa Maria, Frederico Westphalen (FW) campus, 6 isolates were supplied by the UFSM Phytosanitary Defense laboratory (D, DFS), 4 isolates were supplied by the University of Pelotas (Pel), 2 isolates were supplied by the UFSM campus Palmeira das Missões (PM), 1 isolate was supplied by the University of Passo Fundo (PF), 6 isolates were provided by Biota Innovations in the Midwest (BIF), and 5 control isolates were obtained from commercial products already reported as biological nematicides (**Table 1**).

The isolates were kept in Petri dishes containing the Potato-Dextrose-Agar (PDA) medium and incubated at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ in BOD (Biochemical Oxygen

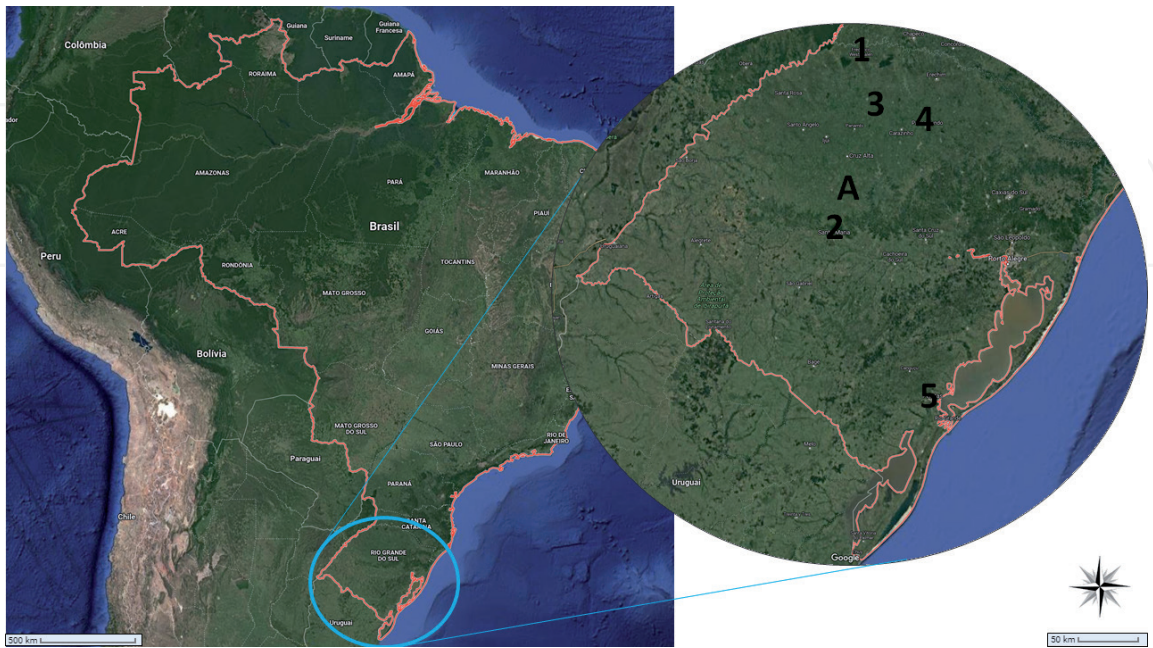


Figure 1. Map of Brazil showing the provided locations of the isolates used in this work. The 1–5 are the cities-state where we obtained *Trichoderma* isolates. The “A” is the location where the nematodes were collected. 1 – Frederico Westphalem-Rio Grande do Sul (RS); 2 – Santa Maria-RS; 3 – Palmeira das Missões-RS; 4 – Passo Fundo-RS; 5 – Pelotas-RS; A – Júlio de Castilhos-RS.

Demand). Each specimen of the fungi was stored in inclined test tubes with PDA medium and kept refrigerated at a temperature of 6 to $10^{\circ}\text{C} \pm 2^{\circ}\text{C}$.

2.2.2 Parasitism test on *M. javanica* eggs

For the parasitism test of the *Trichoderma* isolates on *M. javanica*, eggs of the nematode were extracted manually. These eggs were disinfected so that there was the least possible interference from other microorganisms that could be located on the outside of the eggs. For this, the eggs were placed in a test tube with 0.5% sodium hypochlorite solution, and stirred manually for one minute. After this stage, the eggs were also disinfected with 1% streptomycin and 0.1% 2-mercapto-ethanol (Sigma Aldrich) for four minutes; washed in sterile water and collected with micropipette.

From the obtained suspension, 50 eggs were added, and transferred to individual wells of ELISA plates. In each well, together with the J2, 100 μL of fungal suspension (10^8 conidia / ml) was added. Then the plates were kept in the dark in a BOD under a temperature of $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Evaluations were performed 15 days after application. The numbers of parasitized eggs were determined. This test was repeated twice for greater data reliability.

2.2.3 Obtaining *Trichoderma* filtrates

Each fungus was grown in Petri dishes with PDA culture medium. Seven days after incubation at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$, three disks of 5 mm in diameter were removed from the edges of the cultures and placed in a 250 mL Erlenmeyer flask containing 100 mL of Czapek Dox liquid medium (0.5 g KCl, 1 g of KH_2PO_4 , 2 g of NaNO_3 , 30 g of sucrose, 0.01 g of $\text{FeSO}_4 \cdot \text{H}_2\text{O}$ and 0.5 g of $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ per 1000 mL of distilled water).

Erlenmeyer flasks were sterilized using the autoclave for 30 minutes. A different isolate was placed in each sterile Erlenmeyer. The flasks were kept in an incubator at 25°C with constant agitation for 15 days. After this period, the entire content of each Erlenmeyer was filtered through a cellulose acetate membrane, with an opening of 0.22 μm . For each isolate, the cellulose acetate membrane was exchanged. The fungal filtrates obtained were kept refrigerated for 48 hours at a temperature of 6 to $10^{\circ}\text{C} \pm 2^{\circ}\text{C}$, until the assay was established.

2.2.4 Nematode mortality and hatch inhibition test

For the mortality test of second stage juveniles (J2) of *M. javanica*, the Baermann funnel methodology modified by [14] was followed. The suspension with J2 was obtained from the hatching chamber with a tissue. From this suspension, 50 nematodes were removed through individual capture. In Elisa plates, 20 μL of water were pipetted and added to 80 μL of the fungal filtrates along with 50 captured nematodes. Mortality was assessed 48 hours after the application of the nematode suspension.

For the hatching test, first the egg suspension was obtained according to the methodology of [7]. Then we placed 50 eggs per well of the ELISA plate for the trial. The evaluation was made on the 21st day, when the count of 50 eggs was performed. In each treatment, eight repetitions were performed, kept at 25°C in the dark. These tests were repeated twice, aiming at increasing the data reliability. In the study, two controls were used, one containing only distilled water and the other containing only Czapek Dox medium, to eliminate the possibility of some type of unexplained alteration caused by the Czapek Dox medium that was used to perform the filtrates.

Isolates	Origin (Brazil)*	Treatment code	Species
FW09	South-FW	T1	T. asperellum
FW13	South-FW	T2	T. asperellum
FW14	South-FW	T3	T. virens
FW16	South-FW	T4	T. asperellum
FW21	South-FW	T5	T. asperellum
FW23	South-FW	T6	T. asperellum
FW31	South-FW	T7	T. virens
FW33	South-FW	T8	T. asperellum
FW36	South-FW	T9	T. virens
FW40	South-FW	T10	T. virens
UFMQ1	South-SM	T11	T. virens
PM50	South-PM	T12	T. harzianum
PM63	South-PM	T13	T. harzianum
UFQM14	South-SM	T14	T. harzianum
PF102	South-PF	T15	T. harzianum
D33	South-SM	T16	T. asperellum
DFS03	South-SM	T17	T. virens
DFS04	South-SM	T18	T. asperellum
DFS05	South-SM	T19	T. asperellum
DFS06	South-SM	T20	T. harzianum
DFS07	South-SM	T21	T. asperellum
Pel210	South-Pel	T22	T. asperellum
Pel219	South-Pel	T23	T. harzianum
Pel221	South-Pel	T24	T. asperellum
Pel233	South-Pel	T25	T. harzianum
UFMQ36	South-SM	T26	T. asperellum
UFQM27	South-SM	T27	T. asperellum
BIF0113	Southeast-**	T28	T. asperellum
BIF0111	Southeast-**	T29	T. asperellum
BIF0107	Southeast-**	T30	T. harzianum
BIF0119	Southeast-**	T31	T. asperellum
BIF0162	Southeast-**	T32	T. brevilcompactum
BIF0115	Southeast-**	T33	T. atroviride
UFQM34	South-SM	T34	T. asperellum
UFQM35	South-SM	T35	T. harzianum
CCT-7589	Midwest-**	T36	T. harzianum
SF-04	Midwest-**	T37	T. asperellum
12616	Midwest-**	T38	T. asperellum
T-22	Midwest-**	T39	T. harzianum
ESALQ-1306	Southeast-**	T40	T. harzianum

*FW: Frederico Westphalem-RS; SM: Santa Maria-RS; PM: Palmeira das Missões-RS; PF: Passo Fundo-RS; Pel: Pelotas-RS.
**The city of origin of these isolates are not known or were not disclosed by the provider.

Table 1.
Trichoderma isolates obtained from different regions of Brazil.

2.2.5 Experimental design and statistical analysis

The experimental design used was completely randomized with eight replicates for each treatment, and each fungal isolate corresponded to one treatment (40 isolates). The variables evaluated were: number of live and dead J2 nematodes, count of J2 hatching, count of the number of parasitized and darkened eggs. The results were subjected to analysis of variance, and the means of each treatment were compared by the Scott-Knott cluster test at 5% probability of error, by the SISVAR software [15].

2.2.6 Molecular identification of *Trichoderma* spp.

The total genomic DNA was extracted by the method described by [16]. The *Trichoderma* isolates were placed in a 1.5 mL microtube with 400 µL of extraction solution (Tris-HCl 100 mM pH 8.0; EDTA 20 mM pH 8.0; NaCl 1.4 M; CTAB 2%; PVP 1%; 2-Mercaptoethanol 0.1% and Proteinase K 0.01%) previously heated to 65°C for 3 min. and vortexed for 10 seconds. Then, it was incubated in a water bath at 65°C for 45 minutes, shaking every 15 min.

Then 400 µL of chloroform was added and stirred by gentle inversions for 5 min. Afterwards, it was centrifuged at 14000 rpm, 20°C, for 5 min. After centrifugation, approximately 400 µL of the aqueous phase was removed and transferred to a new 1.5 mL microtube, where 200 µL of chilled isopropanol (2-propanol) was added and homogenized by gentle inversions for 1 minute and incubated at -20°C for 30 min. The solution was centrifuged at 1400 rpm, 20°C, for 5 min. The supernatant was discarded, keeping only the pellet at the bottom of the microtube. For DNA precipitation, 200 µL of cold 70% ethanol (4°C) was added to the tube, followed by centrifugation at 14000 rpm at 4°C, for 5 min. and the supernatant was discarded keeping the pellet formed. The precipitate was dried at room temperature, and recovered in a volume of 50 µL TE [1 mM Tris and 0.1 mM EDTA] + RNase and incubated at 37°C for 30 min., and its DNA was quantified and stored at -20°C until use.

The genomic DNA samples extracted from the fungi were subjected to polymerase chain reaction (PCR) with that performed for partial amplification of the elongation factor gene (EF-1α) with the primers 5'-ATGGGTAAGGARGACAAGAC-3' and 5'-GGARGTACCAGTSATCATGTT3-. For this, 3 µL of the fungi DNA were added to the final volume of the 25 µL PCR reaction, containing 10 mM Tris HCl pH 8.3; 50 mM KCl; 1.1 mM MgCl₂; 10 mM of each dNTP; 25 nmoles of each EF1 and EF2 primer; 1.5 µL of Taq DNA polymerase (Invitrogen, Brazil) and ultrapure water to complete the reaction volume. A negative control without DNA was included in the PCR. The amplification reactions were carried out in a thermocycler (Applied Biosystems 2720, Thermo Fisher Scientific, USA), under the following conditions: 94°C for 1 min., 35 cycles of 95°C for 3 min., 95°C for 1 min., 72°C for 1 min. and 30 seconds, and 72°C for 10 min. At the end of the reaction, the amplified fragments were kept at 4°C. To verify amplification, electrophoresis was performed on 1.5% agarose gel, in TBE 1X buffer, stained with Sybr Gold (Invitrogen, Brazil). PCR products were purified with the Gen Elute PCR clean-up Kit® kit (Sigma, USA) and sequenced (ABI PRISM 3100, Thermo Fisher Scientific, USA). The sequences were analyzed using the Staden Package 2.0.0b program [17] to obtain consensus.

2.2.7 Phylogenetic analysis

The alignment of the nucleotide sequences was performed in the programs Clustal W and Clustal X [18], and sequences deposited in the databases were used

for comparisons. The Neighbor-joining method, using the Jukes-Cantor model, was used to estimate the evolutionary distance. The phylogenetic tree was built in the MEGA X program [19], with the Maximum Likelihood algorithm and the bootstrap values calculated with 1,000 replicates.

3. Results and discussion

3.1 Molecular identification of *Trichoderma* spp.

The *Trichoderma* isolates used in the study were identified from DNA extraction and separated into five distinct species, with 20 isolates belonging to the *T. asperellum* (50% of the isolates) species, 12 isolates of *T. harzianum* (30%), six isolates of *T. virens* (15%), an isolate of *T. brevicompactum* (2.5%) and an isolate belonging to the *T. atroviride* (2.5%) species.

The isolates T36 to T40 are commercially used *Trichoderma* spp. The T36, T39 and T40 were identified as *T. harzianum*, and T37 and T38 as *T. asperellum*. Using these isolates as references we compared with the sequences of other isolates and the clusters that resulted are in the phylogenetic tree (Figure 2).

Analyzing the bootstrap values presented in the dendrogram, we can say that within the clades of the species *T. asperellum*, *T. virens* and *T. harzianum*, we obtained sequences of small size, which caused low bootstrap values, as there was no enough support for the node. In [20], the authors reported that bootstrap values below 70 are normally hidden, and above 70 usually correspond to probabilities greater than 95% that the clade is real.

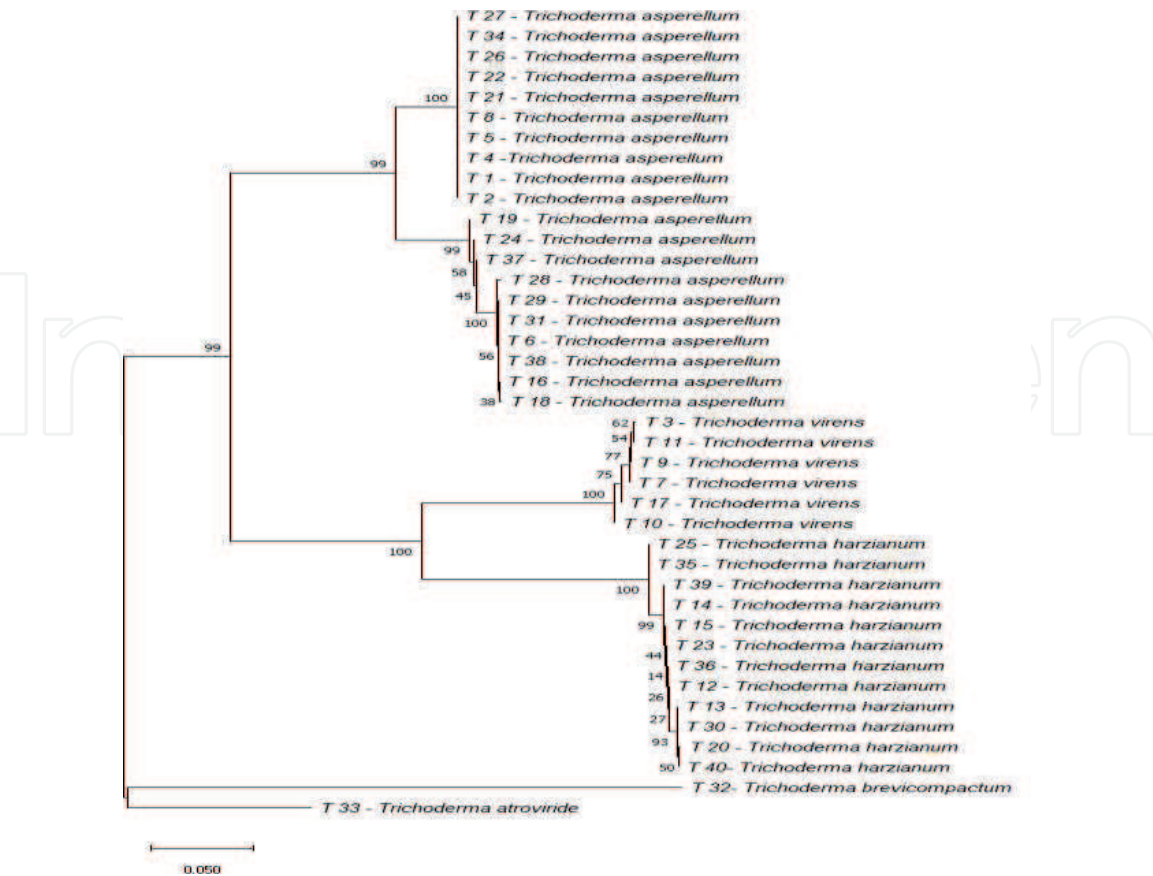


Figure 2.
Dendrogram of the partial sequences of the EF-1 α elongation factor (TEF) gene of *Trichoderma* spp. using the maximum likelihood method based on the Jukes-Cantor model.

3.2 In vitro evaluation of the parasitic, nematocidal and nematostatic effect of *Trichoderma* spp. on *M. javanica*

In this study, it was observed that all species of *Trichoderma* tested showed high potential for suppression of *M. javanica* (**Table 2**). This was confirmed by the effect of parasitism of eggs and nematodes and also by the production of metabolites that killed and/or inhibited the hatching of J2 eggs. The percentage of parasitized J2 nematodes was greater than 85% for all tested isolates, and 35% (14) of the isolates showed a parasitism capacity greater than 95%. The isolates that stood out with the highest parasitism averages, forming a group statistically different from the other isolates, were: *T. harzianum* ESALQ-130 (99%), *T. virens* DFS03 (98.8%), *T. harzianum* UFSM14 (98.7%), *T. atroviride* BIF0115 (98.7%), *T. asperellum* 12616 (98.4%), *T. asperellum* SF-04 (98.4%), *T. harzianum* T-22 (98.3%) %, *T. asperellum* UFSM27 (98.4%), *T. asperellum* BIF0119 (97.9%) and *T. harzianum* PF102 (96.8%).

Regarding the mortality of *M. javanica* using fungal filtrates (**Table 2**), it was observed that 16 (40%) of the isolates had a mortality rate ranging from 85% to 93.1%. Thus, the *Trichoderma* isolates that had the highest mortality values were: *T. harzianum* CCT-7589 (93.1%), *T. brevicompactum* BIF0162 (92.2%), *T. asperellum* BIF0111 (91.9%), *T. atroviride* BIF0115 (91.9%), *T. asperellum* UFSM27 (91.8%), *T. asperellum* BIF0111 (91.7%), *T. harzianum* BIF0107 (91.7%), *T. harzianum* ESALQ-1306 (91.7%), *T. asperellum* UFSM34 (91.6%), *T. harzianum* UFSM35 (91.0%), *T. asperellum* SF-04 (90.9%), *T. asperellum* 12616 (90.8%), *T. harzianum* T-22 (90.6%), *T. harzianum* PM63 (90.1%), *T. virens* DFS03 (88.7%), *T. asperellum* Pel221 (85.8%) and *T. asperellum* BIF0119 (85%). In contrast, the isolates that obtained the lowest percentages of J2 mortality from *M. javanica* were *T. asperellum* FW16 and *T. asperellum* DFS04, with 70.8 and 65.3% mortality, respectively.

Results similar to the present study were obtained by [21], where all the filtrates obtained from the isolates of *Trichoderma* spp. (22 isolates) proved to be efficient in promoting juvenile mortality in a population of *M. incognita* after 24 hours of application of the filtrates in vitro. It is reported that the parasitic effect of fungi of the genus *Trichoderma* against nematodes from egg to adult stages, requires some facilitating mechanism [22], such as the presence of lytic enzymes. The mortality of J2 de Meloidogyne may be related to the presence of enzymes proteases and chitinases that act in the degradation of the cuticle of nematodes, a resistant coating structure composed of proteins and chitin. For this reason, metabolites obtained from fungal isolates that may have these facilitating mechanisms are also sought.

Trichoderma isolates produce metabolites, such as lytic enzymes, which are released into the rhizosphere solute, in our case, culture medium solute. Thus, the inhibition of J2 hatching from the application of *Trichoderma* filtrates may be related to enzymatic activity in the solution. Meloidogyne eggs are composed of approximately 30% chitin in the outer layer, in addition to other structural proteins, and lipids in the inner layer. In this sense, the action of inhibiting the hatching of J2 indicates the presence of enzymes proteases, chitinases and lipases that acted on the enzymatic degradation of the outer and inner layers of the eggs of *M. javanica*, reducing the hatching capacity of J2 of the eggs.

Regarding the nematocidal and nematostatic effect in the inhibition of the hatching of J2 by fungal filtrates, it was observed that 25% of the isolates resulted in a high inhibition of the hatching of *M. javanica* J2, varying from 91.6% to 94.4% hatching inhibition (**Table 2**). The isolates that showed the highest percentage of inhibition statistically were: *T. harzianum* CCT-7589 (94.4%), *T. asperellum* UFSM34 (94.0%), *T. asperellum* SF-04 (93.8%), *T. harzianum* BIF0107 (93.4%), *T. atroviride* BIF0115 (92.6%), *T. brevicompactum* BIF0162 (92.5%), *T. harzianum* T-22 (91.9%), *T. harzianum* UFSM35 (91.8%), *T. harzianum* ESALQ-1306 (91.8%) and

Treatments	Species	J2 parasitized (%)		J2 mortality (%)		J2 hatching inhibition (%)	
T1	Trichoderma asperellum	92.62	C	74.9	C	84.6	C
T2	Trichoderma asperellum	90.87	C	80.4	B	81.5	C
T3	Trichoderma virens	87.81	E	81.7	B	73.1	D
T4	Trichoderma asperellum	91.56	C	70.8	D	84.9	C
T5	Trichoderma asperellum	93.37	C	81.5	B	79.4	C
T6	Trichoderma asperellum	88.12	E	84.3	B	80.6	C
T7	Trichoderma virens	91.25	C	82.5	B	83.5	C
T8	Trichoderma asperellum	87.62	E	77.9	B	86	C
T9	Trichoderma virens	85.5	E	75.9	C	83.6	C
T10	Trichoderma virens	93.37	C	80.3	B	66	E
T11	Trichoderma virens	94.62	B	79.2	B	84.8	C
T12	Trichoderma harzianum	90.43	C	79.4	B	85.3	C
T13	Trichoderma harzianum	89.87	D	90.1	A	87.3	B
T14	Trichoderma harzianum	98.68	A	83	B	87.2	B
T15	Trichoderma harzianum	96.81	A	84.2	B	86.7	B
T16	Trichoderma asperellum	92.5	C	75.5	C	77.7	C
T17	Trichoderma virens	98.81	A	88.7	A	87.2	B
T18	Trichoderma asperellum	95	B	65.3	D	85.3	C
T19	Trichoderma asperellum	93.31	C	74.8	C	85.5	C
T20	Trichoderma harzianum	94.81	B	79.3	B	83.6	C
T21	Trichoderma asperellum	94.18	B	78.9	B	81.5	C
T22	Trichoderma asperellum	95.75	B	80.2	B	79.9	C
T23	Trichoderma harzianum	92.62	C	80.8	B	79.7	C
T24	Trichoderma asperellum	94.06	B	85.8	A	88.7	B
T25	Trichoderma harzianum	94.87	B	81.2	B	85.2	C
T26	Trichoderma asperellum	92.18	C	82.4	B	83.8	C
T27	Trichoderma asperellum	98.43	A	91.8	A	88.3	B
T28	Trichoderma asperellum	89.75	D	74.8	C	82.9	C
T29	Trichoderma asperellum	89.68	D	91.9	A	88.5	B
T30	Trichoderma harzianum	93.93	B	91.7	A	93.4	A
T31	Trichoderma asperellum	97.93	A	85	A	87.2	B
T32	Trichoderma brevicompactum	92.62	C	92.2	A	92.5	A
T33	Trichoderma atroviride	98.68	A	91.9	A	92.6	A
T34	Trichoderma asperellum	96.18	B	91.6	A	94	A
T35	Trichoderma harzianum	94.12	B	91	A	91.8	A
T36	Trichoderma harzianum	95.68	B	93.1	A	94.4	A
T37	Trichoderma asperellum	98.43	A	90.9	A	93.8	A
T38	Trichoderma asperellum	98.43	A	90.8	A	91.6	A
T39	Trichoderma harzianum	98.31	A	90.6	A	91.9	A

Treatments	Species	J2 parasitized (%)		J2 mortality (%)		J2 hatching inhibition (%)	
T40	Trichoderma harzianum	99	A	91.7	A	91.8	A
Control H ₂ O*		0	F	6.3	E	8	F
Control Czapek Dox**		—		5	E	7.1	F
CV (%)		4.37		6.9		7.8	
Means followed by different capital letters are differentiated by the Scott-Knott hierarchical cluster test with 95% confidence.							
*Only used H ₂ O as control treatment.							
**Only used Czapek Dox medium as control treatment.							

Table 2.
Effect of parasitism of *Trichoderma* spp. and nematicidal and nematostatic effect of metabolites of fungal filtrates in *Meloidogyne javanica*.

T. asperellum 12616 (91.6%). In contrast, the isolate *T. virens* FW40 resulted in the lowest inhibition capacity of J2 hatching (66% inhibition).

In [23], they concluded that two *Trichoderma* isolates (*T. asperellum* M2RT4 and *Trichoderma* sp. MK4) significantly reduced the number of hatched J2s, between 60.8 and 81.8%. In the same work, it was observed that *T. asperellum* M2RT4 was the most efficient isolate for the control of galls, egg mass and deposited eggs, reducing, respectively, 81.8, 78.5 and 88.4%, indicating that isolates from this species have potential for biocontrol. Both results coincide with data on egg and J2 mortality and inhibition of hatching of J2, obtained in this work. On the other hand, *T. atroviride* F5S21 had no significant effect in comparison to the control, which goes against the result obtained in the present study, whereas *T. atroviride* (T33) showed positive results in parasitism, mortality and inhibition of hatching of J2.

The authors [24] mention that *Trichoderma* filtrates have toxic effect on adults of *Meloidogyne* sp. The same was observed by [25], in which all filtrates showed toxic activity against *M. incognita*, obtaining 98% of immobile and dead J2.

In a study by [26] with the objective of evaluating the effect of *T. harzianum* for the biological control potential of *M. javanica*, they also found promising results for nematode suppression. In this study, they also evaluated the metabolite production capacity and its relationship to nematode suppression. The authors concluded that the increased activity of the chitinase enzyme is directly related to the capacity of suppression of *M. javanica* by the species *T. harzianum*.

The production of lytic enzymes also helps in the penetration of the fungus, especially visualized in *T. harzianum* [27, 28]. The ability to produce metabolites, as we can see, is also an important factor in the suppression of nematodes [29]. In our study, the production of metabolites by fungi, through fungal filtrates, also had a positive result for biocontrol of *M. javanica*. The production of metabolites by the fungi inhibited the outbreak of J2, and the isolate that obtained the lowest percentage of inhibition of J2 was a *T. virens* isolate. However, we can observe that this same species had percentages above 80% of mortality, parasitism and inhibition of hatching, which shows us an important difference between the *Trichoderma* isolates.

Results from [30] showed that, fungi of the *Trichoderma* genus, specifically *T. harzianum* in this study, presented positive results for the mortality and hatch inhibition of J2 comparing with control, *Paecilomyces lilacinus* and humic acid. And [31], also observed for 3 different isolates of *Trichoderma* 100% larval mortality for *M.*

javanica and between 94,1 and 100% larval mortality of *M. incognita*. Both studies support our findings for the potential of *Trichoderma* for *Meloidogyne* mortality.

As we can see in the results of this work and supported by others, there is a significant difference in the suppression of nematodes among isolates of the same species. We observed in our work species of *T. asperellum* that ranged from 87.62% of parasitized J2, up to 98.43%, and also, other strains of the same species showed 65.3% of J2 mortality, while others showed 91.9%, and for the percentage of inhibition of J2 hatching, there was a variation from 77.7–94%, within the same species. In the species *T. harzianum*, there were variations of 89.87–99% in parasitism, of 79.3% to 93.1% of J2 mortality from the filtrates, and from 79.7% to 94.4% of hatching inhibition, in different strains.

This is due to the fact that each strain has a different gene expression, that is, it has genetically the ability to produce different lytic enzymes [22, 32], or it has the parasitic capacity expressed by virulence genes. For this reason, selections of highly efficient strains are carried out in the biocontrol of pests and diseases.

It was tested by [12] 230 isolates, and only 8 belonging to the South region. All organisms obtained in prospecting processes should be tested as potential antagonistic agents for different pathogens and pests. The authors affirm the importance of prospecting for biological control agents and characterizing them, mainly fungi, in the entire Brazilian territorial area, since good antagonists may be dispersed in different regions of the country. This work shows the potential of five species of *Trichoderma* in the biological control of *M. javanica*, and future experiments in natural conditions could help to widen the differences of each of the studied strains.

4. Conclusion

All *Trichoderma* isolates tested showed potential for *Meloidogyne javanica* biocontrol.

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Conflict of interest

The authors hereby declare no conflict of interest.

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