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ORIGINAL ARTICLE

Suppression of the phytopathogens *Sclerotinia sclerotiorum* and *Sclerotium rolfsii* by *Trichoderma* spp.

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Abstract

This work was carried out to evaluate the antagonism of *Trichoderma* isolates to the soil-borne plant pathogenic fungi *Sclerotinia sclerotiorum* and *Sclerotium rolfsii*. Thirty-four antagonist fungal strains were used. In the dual culture tests, *Trichoderma* isolates CEN281 and CEN287 stood out, both belong to the species *T. afroharzianum* and originate from soil cultivated with cotton. The folding, deformation and hyphal penetration effects of the pathogens were confirmed under microscopy in all the *Trichoderma* isolates. In the volatile metabolites production assays, no inhibition of *S. sclerotiorum* growth was observed. On the other hand, six isolates were able to inhibit *S. rolfsii*, including CEN1075 (*T. asperellum*) which originates from the tomato rhizosphere. As for the production of non-volatile metabolites, 11 isolates, mostly obtained from soil samples from the tomato rhizosphere, showed action against both pathogens. Regarding the damping-off of tomato seedlings caused by *S. rolfsii*, it was observed that the *Trichoderma* isolates CEN281 and CEN126 were the ones that most suppressed the disease, and both were isolated from the rhizosphere of this plant. This work allowed the selection of new *Trichoderma* isolates with potential antagonistic to the important soil fungi in question. The isolates CEN281, CEN1070 and CEN1080 showed the best results in controlling the damping-off of tomato seedlings and, therefore, will be evaluated in field studies.

Keywords Antagonist fungi · Biological control · Damping-off · Soil-borne fungi · *Sclerotium* stem rot

Introduction

The tomato plant (*Solanum lycopersicum* L.), belonging to the *Solanaceae* family, is probably native to the Andean Region, western part of South America; was domesticated in Mexico and distributed to other regions of the globe (Knapp and Peralta 2016), until reaching Brazil. Among the vegetables, the tomato stands out, due to the volume of production and jobs generated in its production (Machado et al. 2018). However, to meet the market demand for new products, intensive research work is required to select efficient antagonists against soil-borne pathogens, including *Sclerotinia sclerotiorum* (Lib.) de Bary - white mold, and *Sclerotium rolfsii* Sacc. - sclerotium rot (Singh et al. 2017).

Plant diseases are among the main causes of agricultural losses worldwide and their management is a challenge in the food security of the world population (Nelson 2020). Several approaches have been recommended to prevent or mitigate the effects caused by plant pathogens, including

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agronomic and sanitation practices (Thambugala et al. 2020; Asad 2022). The experience of controlling plant diseases, with the exclusive use of chemical fungicides, has already proved to be unfeasible, especially in the case of soil-borne pathogens (Smolińska and Kowalska 2018), in addition, such products can also have negative effects on the health of consumers and rural workers, damage the environment and, over time, result in resistance to these products (Zubrod et al. 2019). This fact further aggravates the health problems for which such products are indicated. To combat this menace, use of biological control agent for the management of plant diseases is the best possible alternative (Akhtar and Javaid 2018).

Trichoderma is a genus of anamorphic fungi, with the sexual (teleomorphic) phase in *Hypocrea* (Hypocreales, Ascomycota), originally introduced by Persoon (1794). These fungi have outstanding importance as bioregulators and natural antagonists of soil-borne phytopathogenic fungi, due to their great competitive capacity and a diversity of mechanisms of action (Benítez et al. 2004; Jaiswa and Khadk 2020). The growing concern about the effects of synthetic fungicides on the environment and the residues of such products in food has reinforced the interest in the use of fungi of the *Trichoderma* genus in agriculture (Abdelkhalek et al. 2022) and study of its occurrence (Silva et al. 2020a, b). In Brazil, according to the platform of the Ministry of Agriculture, Livestock and Supply, there are currently about 58 *Trichoderma*-based products registered as biofungicides (Agrofit 2023). It is important to note that some of the isolates used as active ingredients in these products are repeated under different trade names. The variations of products with the same isolates are caused by changes in the formulation, target and concentration of spores, therefore, it is important to search for isolates from different species for use in the composition of new bioproducts.

In the routine for selection of *Trichoderma* isolates for biological control, it is necessary to carry out in vitro tests in paired culture, in addition to verifying the production of volatile and non-volatile metabolites with antimicrobial activity and verification of hyperparasitic interactions by microscopic examination (Asad et al. 2022). Data related to the dual cultures of *S. rolfsii* and *S. sclerotiorum* with *Trichoderma* spp. have been published by several authors, such Marques et al. 2016, 2022b; Mesquita et al. (2017); Sumida et al. (2018), and by Kamel et al. (2020). Regarding studies on the production of volatile and non-volatile metabolites that are active against these two pathogens, the works by Louzada et al. (2016); Mesquita et al. (2017); Kushwaha et al. (2018); Marques et al. 2018, 2022a; Silva et al. (2020a, b, 2021) stand out. Regarding the verification of the hyperparasitism of *Trichoderma* isolates to phytopathogenic fungi, the works of Louzada et al. (2009), Troian et al. (2014), and by Kamel et al. (2020) are of interest. In vitro studies are useful to verify the mechanism of action of antagonists and to distinguish isolates for in vivo studies, aimed at the selection of potential biocontrol agents (BCAs) and possible use in commercial formulations.

Studies have already linked the use of fungi of the genus *Trichoderma* in the in vivo control of diseases caused by pathogens in tomato fields (Abdelkhalek et al. 2022). Regarding studies with *Trichoderma* in the control of sclerotium rot in tomato, it can be mentioned those carried out by Islam et al. (2016), Suriyagamon et al. (2018), Kamel et al. (2020) and Blanco et al. (2021). However, there are no recommended products for this pathogen in tomato crops in Brazil (Agrofit 2023). Keeping in view of the importance of routine studies of prospection and characterization of *Trichoderma* isolates, mainly for soil-borne and difficult-to-control pathogens, the main objective of this work was to isolate, select and evaluate fungi of the genus *Trichoderma* *in vitro* against the pathogens that cause white mold and sclerotium rot *in vivo.*

Materials and methods

Isolates from pathogens and antagonists

The work was carried out at the Phytopathology Laboratory of the Biological Control Building of Embrapa Recursos Genéticos e Biotecnologia (CENARGEN), Brasília-DF (Distrito Federal), Brazil. Thirty-four *Trichoderma* isolates were used, of which 13 were obtained from soil samples collected in a tomato cultivation area at Embrapa Hortaliças (CNPH), located in Gama (DF), and the other isolates were already deposited in the Collection of Fungi for Biological Control of Embrapa Recursos Genéticos e Biotecnologia. The other 21 isolates were selected based on preliminary results obtained in the control of white mold (*S. sclerotiorum*) and bean collar rot (*S. rolfsii*) by other team members. Among the isolates used, ten were identified at the species level, as shown in Table 1, and some were characterized in a previous work regarding growth promotion in tomato plants (Montalvão et al. 2020).

In the biocontrol assays, isolates CEN217 and CEN216 of *S. sclerotiorum* and *S. rolfsii* were used, respectively. These are also stored in the Embrapa collection.

Dual culture assessment

Antagonistic potential was assessed by the paired culture technique (Dennis and Webster 1971a). Isolates from both pathogens and antagonists were multiplied in PDA (Potato-Dextrose-Agar) medium at 25ºC and 12 h of photoperiod,

Table 1 Description of *Trichoderma* isolates used in this study

Embrapa Number	Identification	Isolation Source	Collection Location
CEN126	*Trichoderma* sp.	Cacao	----
CEN129	*Trichoderma* sp.	Cacao	----
CEN141	*T. afarasin*	Soybean	Orizona-GO
CEN155	*Trichoderma* sp.	Maize	Orizona-GO
CEN161	*Trichoderma* sp.	Rice	Goianira-GO
CEN162	*T. asperelloides*	Rice	Goianira-GO
CEN169	*Trichoderma* sp.	Forage sorghum	Rio Verde-GO
CEN170	*Trichoderma* sp.	Maize	Rio Verde-GO
CEN194	*Trichoderma* sp.	----	----
CEN201	*Trichoderma* sp.	Vochysiaceae	Goiatins/ Itacará-MT
CEN209	*T. pseudokoningii*	Copaifera	Planaltina-DF
CEN219	*T. afroharzianum*	----	----
CEN223	*Trichoderma* sp.	Cotton	----
CEN273	*Trichoderma* sp.	Cotton	Rio Preto-DF
CEN280	*T. longibrachiatum*	----	----
CEN281	*T. afroharzianum*	Cotton	Rio Preto-DF
CEN287	*T. afroharzianum*	Cotton	Rio Preto-DF
CEN288	*Trichoderma* sp.	Cotton	Rio Preto-DF
CEN289	*Trichoderma* sp.	Cotton	Rio Preto-DF
CEN290	*Trichoderma* sp.	Cotton	Rio Preto-DF
CEN316	*Trichoderma* sp.	Cotton	Rio Preto-DF
CEN1068	*Trichoderma* sp.	Tomato	CNPH/Gama-DF
CEN1069	*Trichoderma* sp.	Tomato	CNPH/Gama-DF
CEN1070	*Trichoderma* sp.	Tomato	CNPH/Gama-DF
CEN1071	*T. brevicompactum*	Tomato	CNPH/Gama-DF
CEN1072	*Trichoderma* sp.	Tomato	CNPH/Gama-DF
CEN1073	*Trichoderma* sp.	Tomato	CNPH/Gama-DF
CEN1074	*T. brevicompactum*	Tomato	CNPH/Gama-DF
CEN1075	*T. asperellum*	Tomato	CNPH/Gama-DF
CEN1076	*Trichoderma* sp.	Tomato	CNPH/Gama-DF
CEN1077	*Trichoderma* sp.	Tomato	CNPH/Gama-DF
CEN1078	*Trichoderma* sp.	Tomato	CNPH/Gama-DF
CEN1079	*Trichoderma* sp.	Tomato	CNPH/Gama-DF
CEN1080	*Trichoderma* sp.	Tomato	CNPH/Gama-DF

for seven days. For this experiment, agar discs colonized by fungi were used, which were placed on opposite sides of the plates. As a control treatment, PDA plates containing only the pathogen were prepared. For each *Trichoderma* isolate, three replications were performed. The evaluations were carried out at seven days of cultivation, assigning scores according to the scale of note discribed by Bell et al. (1982).

The structures of the confrontation zones of the two fungi were analyzed with the aid of an Optical Microscope (OM) (Olympus BX40), with a 40X magnifying glass. For that, slides containing fragments of these structures of confrontation between the fungi were made and observed for the presence of curling, hyphal plasmolysis, growth of parallel hyphae, as well as structural alterations of these hyphae.

A completely randomized design was used, with three replications. They were performed twice.

Antagonistic interactions under the scanning electron microscope

Detailed studies of the interaction between *Trichoderma* isolates and pathogens were carried out by Scanning Electron Microscopy (SEM), using samples from paired culture, with 11 isolates of the antagonist. For this purpose, discs of PDA (5 mm in diameter) from areas of confrontation between colonies of isolates of *Trichoderma* spp. and pathogens (*S. sclerotiorum* and *S. rolfsii*) were removed and submitted to the procedure described by Bossola and Russell (1998), adapted by Alves (2004).

Volatile metabolites production

To evaluate the inhibitory effect of volatile metabolites, tests were performed according to the methodology described by Silva et al. (2020a, b). Where two plates were overlapping, the bottom one with *Trichoderma* and the top one with the phytopathogen, after 7 h of previous cultivation. To prevent gases from leaking out, the overlapping plates were tightly sealed with PVC film. Incubation was carried out in the same way as described above. The control treatment were plates with PDA containing the pathogen and another without the antagonist. Mycelial growth assessments were performed when the entire surface of the medium in the control plates, that is, in those whose lower bases contained PDA medium, were covered by the pathogen.

The tests were carried out in a completely randomized design, with three replications and twice.

Non-volatile metabolites production

Tests to evaluate the inhibition of the growth of the pathogen by non-volatile metabolites were performed based on the methodology described by Carvalho et al. (2021), with modifications. For this test, the antagonist isolates were grown in Erlenmeyer flasks (250 mL) containing 100 mL of potato-dextrose-based liquid medium. As inoculum, 5 agar discs (5 mm in diameter) were used per plate, taken from cultures of *Trichoderma* grown in PDA, with five days of age. Incubation was performed in an orbital shaker, at 150 rpm, at a temperature of 25 ± 2ºC, in the absence of light. After seven days, the liquid part was collected by filtration through filter paper and was filtered again with the aid of cellulose membranes of 0.45 µm. The filtrate thus sterilized was incorporated into the autoclaved PDA medium at a rate

of 25% (v/v). For each *Trichoderma* filtrate, three plates were prepared. Petri dishes with the medium received a culture disc of the pathogen at the center and were then kept at 25 ± 2 °C. As a control, plates with PDA medium without the filtered cultures of the antagonist were used. In this experiment, radial mycelial growth measurements were also performed only when the control plate had been completely taken up by the phytopathogen.

The tests were carried out in a completely randomized design, with three replications and twice.

Suppression of tomato seedlings damping-off in greenhouse

The experiment was carried out in a greenhouse maintained at a temperature of 14–35 ºC and humidity around 80%, with a photoperiod of 12 h. *Trichoderma* isolates selected in the previous assays were cultivated in PDA medium at a temperature of 25 ± 2°Cin the presence of light for seven days (Table 2). Culture spores were extracted by adding 20 ml of aqueous Tween 80 solution (0.05) to Petri dishes. The spore suspensions obtained were adjusted to a concentration of 6×10^7 conidia mL^{-1}. The multiplication of the pathogens was carried out according to a methodology adapted from Serra and Silva (2005).

'San Vito' tomato seeds were microbiolized with *Trichoderma* isolates and allowed to germinate in gerbox boxes placed in an incubator with a 12-hour photophase and a temperature of 25 ± 2 °C. Controls were also pre-germinated under the same conditions as above, but without microbiolization with *Trichoderma* isolates.

Table 2 Evaluation of *Trichoderma* isolates when controlling tomato seedling damping-off, based on the average silhouette length method

Strain	Identification	Group	Number of plants without damping-off	Silhouette Validation
CEN1070	*Trichoderma* sp.	1	5.00 ± 0.00	1.00
CEN126	*Trichoderma* sp.	1	5.00 ± 0.00	1.00
CEN281	*T. afroharzianum*	1	5.00 ± 0.00	1.00
CEN1080	*Trichoderma* sp.	1	5.00 ± 0.00	1.00
Control 1	----	1	5.00 ± 0.00	1.00
CEN1071	*T. brevicompactum*	2	4.67 ± 0.48	1.00
CEN1072	*Trichoderma* sp.	2	4.67 ± 0.48	1.00
CEN1073	*Trichoderma* sp.	2	4.67 ± 0.48	1.00
CEN1074	*Trichoderma* sp.	2	4.67 ± 0.48	1.00
CEN1078	*Trichoderma* sp.	2	4.67 ± 0.48	1.00
CEN1079	*Trichoderma* sp.	2	4.67 ± 0.48	1.00
CEN287	*T. afroharzianum*	2	4.67 ± 0.48	1.00
CEN1076	*Trichoderma* sp.	4	4.33 ± 0.52	1.00
CEN1077	*Trichoderma* sp.	4	4.33 ± 0.52	1.00
Control 2	----	3	3.50 ± 0.55	0.58
CEN129	*T. harzianum*	3	3.50 ± 0.55	0.58

After producing antagonist, pathogen and host, the assay was set up as follows: 400 mL of autoclaved soil packed in disposable cups received 20 mL of conidia suspension (6×10^7 conidia mL^{-1}) and, 48 h later, 4 g of rice colonized by the pathogen. Three days later, five germinated tomato seeds were distributed per cup. Two control treatments were included, one with the pathogen and without *Trichoderma* (control 1) and the other without the pathogen and without *Trichoderma* (control 2). After 11 and 17 days of cultivation, the growth of tomato plants, the mortality rate and the presence of lesions were evaluated. For each *Trichoderma* isolate, three replicates were used.

The experimental design was completely randomized, with three replications (pots with five tomato seedlings). The experiments were performed twice.

Silhouette validation method

The Silhouette Validation Technique, according to Rousseeuw (1987), started from the premise by which the silhouette length is calculated for each sample, its average width for each cluster, its global average and the average for the total data set. Thus, with this approximation, each cluster can be represented by the so-called silhouette, based on the comparison of its narrowing and separation. The mean silhouette length was applied to validate the cluster and decide the ideal number of clusters.

The silhouette S(i) construction is given as:

$$S\left(1\right) = \frac{\left(b\left(i\right) - a\left(i\right)\right)}{\max\left\{a\left(i\right), b\left(i\right)\right\}},$$

where a(i) is the average dissimilarity of object i in relation to all other objects in the same cluster; (bi) is the minimum average dissimilarity of object i in relation to all other clusters (in the closest cluster).

It is followed by the formula $-1 \leq S(i) \leq 1$. If the silhouette value is close to 1, it indicates that the sample is well clustered and it has been assigned an opportune cluster. If its value is close to zero, it means that the sample could be allocated to another cluster closer, and it is equally distant from both clusters. If the silhouette value is -1, it indicates that the sample was misclassified. The average silhouette length for all objects is simply the average of S(i).

Statistical analyzes were performed using the "R" Statistical Program.

Results

Dual culture assessment

Regarding the in vitro tests against *S. sclerotiorum*, seven groups of isolates were formed (Table 3), where the mean silhouette length was 0.94, indicating a good separation. In this case, the best inhibition result was observed for group 7, where isolate CEN219 is allocated, followed by the isolates of group 6, which comprises CEN141, CEN223, CEN281 and CEN287. Group 1 was the third best inhibitor and accommodates isolates CEN1068, CEN155, CEN161, CEN170 and CEN273. Group 2 consisted of isolates CEN1069, CEN1071, CEN1074, CEN126, CEN194, CEN280 and CEN289 and was followed by group 3 (CEN1070, CEN1073, CEN1075, CEN129, CEN162 and CEN201); next, appear group 5 (CEN1077, CEN1078, CEN169, CEN209, CEN290 and CEN316) and group 4 (CEN1072, CEN1076, CEN1079, CEN1080 and CEN288), which presented the least satisfactory results, although differing from the control that formed group 8.

Concerning the inhibition of *S. rolfsii* in paired culture, seven groups of isolates were also formed, as shown in Table 3. The mean silhouette length was 0.91, which indicates

Table 3 Grouping of *Trichoderma* isolates regarding growth inhibition of the pathogens *Sclerotinia sclerotiorum* and *Sclerotium rolfsii*, based on the mean silhouette length method

Strain	*Sclerotinia sclerotiorum* inhibition			*Sclerotium rolfsii* inhibition		
	Group	Note according to the Bell scale	Silhouette Validation	Group	Note according to the Bell scale	Silhouette Validation
CEN1068	1	1.67 ± 0.49	1	1	1.33 ± 0.49	1
CEN1069	2	2.00 ± 0.45	1	2	1.00 ± 0.00	1
CEN1070	3	2.33 ± 0.59	1	3	1.67 ± 0.48	1
CEN1071	2	2.00 ± 0.45	1	2	1.00 ± 0.00	1
CEN1072	4	3.00 ± 0.00	1	4	2.67 ± 0.52	1
CEN1073	3	2.33 ± 0.59	1	3	1.67 ± 0.48	1
CEN1074	2	2.00 ± 0.45	1	3	1.67 ± 0.48	1
CEN1075	3	2.33 ± 0.59	1	5	2.00 ± 0.00	1
CEN1076	4	3.00 ± 0.00	1	3	1.67 ± 0.48	1
CEN1077	5	2.67 ± 0.49	1	1	1.33 ± 0.49	1
CEN1078	5	2.67 ± 0.49	1	3	1.67 ± 0.48	1
CEN1079	4	3.00 ± 0.00	1	5	2.00 ± 0.00	1
CEN1080	4	3.00 ± 0.00	1	5	2.00 ± 0.00	1
CEN126	2	2.00 ± 0.45	1	1	1.33 ± 0.49	1
CEN129	3	2.33 ± 0.59	1	2	1.00 ± 0.00	1
CEN141	6	1.33 ± 0.49	1	1	1.33 ± 0.49	1
CEN155	1	1.67 ± 0.49	1	3	1.67 ± 0.48	1
CEN161	1	1.67 ± 0.49	1	3	1.67 ± 0.48	1
CEN162	3	2.33 ± 0.59	1	3	1.67 ± 0.48	1
CEN169	5	2.67 ± 0.49	1	2	1.00 ± 0.00	1
CEN170	1	1.67 ± 0.49	1	2	1.00 ± 0.00	1
CEN194	2	2.00 ± 0.45	1	2	1.00 ± 0.00	1
CEN201	3	2.33 ± 0.59	1	5	2.00 ± 0.00	1
CEN209	5	2.67 ± 0.49	1	5	2.00 ± 0.00	1
CEN219	7	1.00 ± 0.00	0	5	2.00 ± 0.00	1
CEN223	6	1.33 ± 0.49	1	4	2.67 ± 0.52	1
CEN273	1	1.67 ± 0.49	1	2	1.00 ± 0.00	1
CEN280	2	2.00 ± 0.45	1	5	2.00 ± 0.00	1
CEN281	6	1.33 ± 0.49	1	2	1.00 ± 0.00	1
CEN287	6	1.33 ± 0.49	1	2	1.00 ± 0.00	1
CEN288	4	3.00 ± 0.00	1	6	2.33 ± 0.58	0
CEN289	2	2.00 ± 0.45	1	2	1.00 ± 0.00	1
CEN290	5	2.67 ± 0.49	1	7	3.00 ± 1.00	0
CEN316	5	2.67 ± 0.49	1	1	1.33 ± 0.49	1
Control	8	5.00 ± 0.00	0	8	5.00 ± 0.00	0

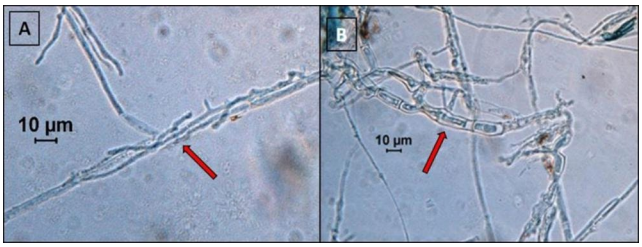

Fig. 1 OM images. (**A**) – Red arrow indicating coiling of hyphae of isolate CEN169 (*Trichoderma* sp.) in hyphae of CEN217 (*S. sclerotiorum*); (**B**) – Arrow indicating deformation of pathogen hyphae

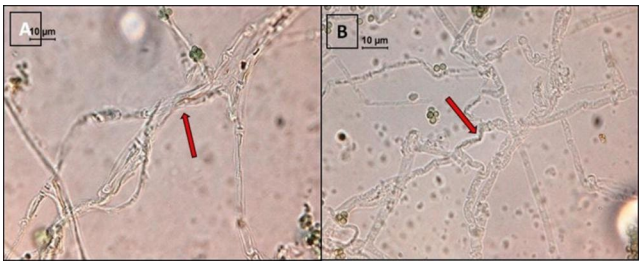

Fig. 2 OM images. (**A**) – Red arrow indicating hyphal coiling of isolate CEN169 (*Trichoderma* sp.) in hyphae of CEN216 (*Sclerotium rolfsii*); (**B**) – Arrow indicating deformation of pathogen hyphae

a good separation of the groups. According to the silhouette method, the tested isolates were divided into groups according to their efficiency in inhibiting the mycelial growth of the pathogen, where the best result was achieved by group 2 composed of isolates CEN1069, CEN1071, CEN129, CEN169, CEN170, CEN194, CEN273, CEN281, CEN287 and CEN289; the second best result was obtained by group 1, which comprises isolates CEN1068, CEN1077, CEN126, CEN141 and CEN316; in third place was group 3, where the isolates are CEN1070, CEN1073, CEN1074, CEN1076, CEN1078, CEN155, CEN161 and CEN162, followed by group 5 (CEN1075, CEN1079, CEN1080, CEN201, CEN209, CEN219 and CEN280), group 6 (CEN288), group 4 (CEN1072 and CEN223), group 7 (CEN290) and finally group 8, formed only by the control treatment.

Interaction of pathogens and *Trichoderma* spp. under optical and scanning electron microscopy

In the dual culture carried out between the 34 isolates of *Trichoderma* and isolates CEN217 (*S. sclerotiorum*) and CEN216 (*S. rolfsii*), antagonistic interactions were observed, typically due to competition and hyperparasitism. As a result of competition, there was entanglement and deformation of hyphae (Figs. 1 and 2), as observed in light microscopy. It was also observed in some cases that *Trichoderma* isolates penetrated and colonized propagules (sclerotia) of *S. sclerotiorum* and *S. rolfsii* isolates.

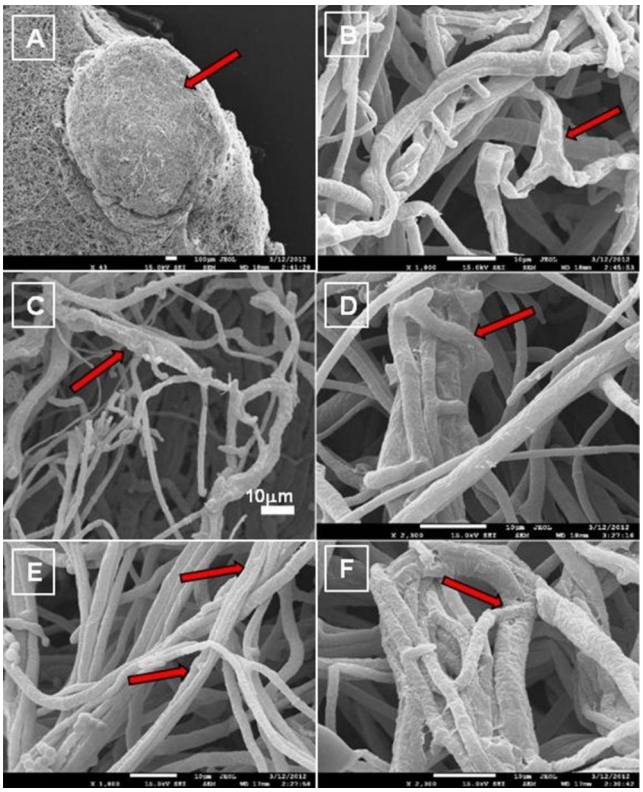

Fig. 3 SEM images. (**A**) – Rede arrow pointing to sclerotia of *Sclerotinia sclerotiorum* (CEN217); (**B**) – Arrow indicating deformed hyphae of the pathogen; (**C**) – Arrow indicating hypha of *Sclerotium rolfsii* (CEN216) being parasitized by *Trichoderma* sp. (CEN1073 isolate); (**D**) – Arrow indicating hyphal coiling of the isolate CEN287 (*Trichoderma afroharzianum*) in *S. rolfsii*; (**E**) – Arrows indicating hyphae with appressoria of isolate CEN129 (*Trichoderma* sp.) on CEN216 and (**F**) – Arrow indicating hypha penetration of isolate CEN129 (*Trichoderma* sp.) in hypha of *S. rolfsii*

Due to the impossibility of carrying out observations using a scanning electron microscope (SEM) with all the tests performed, 11 of them were chosen, involving both the pathogens *S. sclerotiorum* (CEN217) and *S. rolfsii* (CEN216), with nine isolates of *Trichoderma*. The clashes analyzed were: CEN217 x *Trichoderma* sp. (isolate CEN129); CEN217 x *Trichoderma* sp. (isolate CEN155); CEN217 x *Trichoderma* sp. (isolate CEN201); CEN217 x T. *asperellum* (isolate CEN1075); CEN216 x *Trichoderma* sp. (isolate CEN129); CEN216 x *Trichoderma* sp. (isolate CEN155); CEN216 x T. *afroharzianum* (isolate CEN281); CEN216 x T. *afroharzianum* (isolate CEN287); CEN216 x *Trichoderma* sp. (isolate CEN289); CEN216 x T. *brevicompactum* (isolate CEN1071) and CEN216 x *Trichoderma* sp. (isolate CEN1073).

The SEM showed the parallel growth and intertwining of hyphae of the pathogens and antagonists (Figs. 3 and 4), hydrolysis and deformation of the hyphae of the pathogens by the antagonists, as well as the penetration of *Trichoderma*

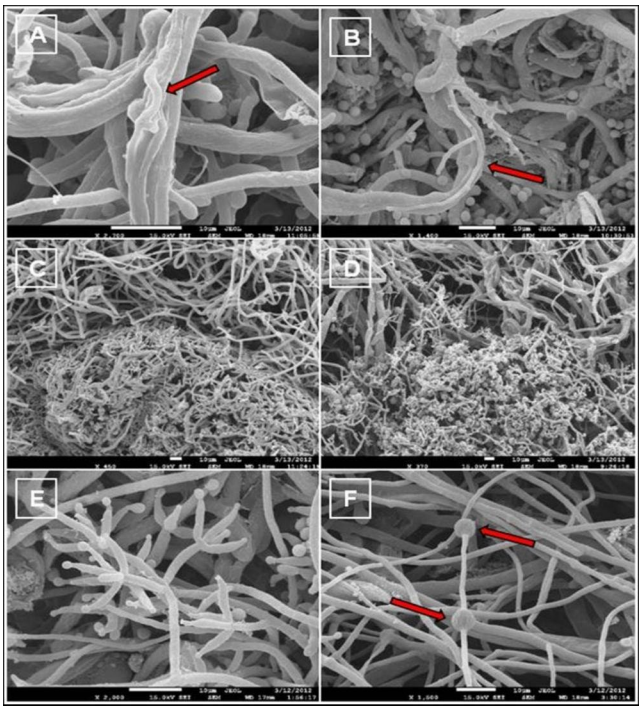

Fig. 4 SEM images. (**A**) – Rede arrow indicating hyphae of the *Trichoderma* sp. (CEN155) parasitizing *Sclerotinia sclerotiorum* (CEN216); (**B**) – Arrow indicating the growth of hyphae of *T. asperellum* (isolate CEN1075) parallel to hyphae of the same pathogen; (**C**) – view of the confrontation region between *Trichoderma* sp. (tangle of hyphae more compacted at the base of the image) *S. sclerotiorum* (tangle of hyphae looser in the upper area of the image); (**D**) – View of the confrontation region between CEN281(*T. afroharzianum*) at the base of the image and CEN216 (*S. rolfsii*) in the upper area of the image; (**E**) – Reproductive structures of *Trichoderma* (CEN201), with its ampuliform phialid conidiogenic cells and smooth unicellular conidia; (**F**) – Arrows indicating intercalated chlamydospores of *T. afroharzianum* (CEN287)

spp., proving the hyperparasitism exerted by different *Trichoderma* isolates on *S. sclerotiorum* and *S. rolfsii*.

Volatile metabolites

Of the 34 *Trichoderma* isolates tested for volatile metabolites production, none showed an apparent influence on the growth of *S. sclerotiorum*.

On the other hand, the results obtained in the assays for the production of active volatile metabolites against *S. rolfsii* led to the formation of three inhibition groups, as shown in Table 4. The mean silhouette length was 0.59, which indicates a reasonable separation of the groups of isolates. In accordance with Table 4, for this experiment, the best results were achieved with the isolates from group 3, which comprises nine isolates: CEN126, CEN155, CEN170, CEN169, CEN129, CEN290, CEN219, CEN1075 and CEN273, followed by group 1 with 14 isolates: CEN141, CEN201, CEN161, CEN1074, CEN289, CEN1069, CEN288, CEN209, CEN281, CEN194, CEN1068, CEN1073, CEN1079 and CEN162 and group 2 formed by 11 isolates: CEN1072, CEN1076, CEN316, CEN223, CEN1078, CEN1080, CEN1071, CEN287, CEN1077, CEN1070 and CEN280; finally, group 4 was constituted by the control. All groups of *Trichoderma* isolates used in this assay differed significantly from the control in terms of reducing the mycelial growth of *S. rolfsii* by the production of volatile compounds. Colonies of the phytopathogen reached lower growth than the control, which reached 9.0 cm in diameter, meaning that the antagonist isolates under study produce volatile toxic compounds capable of inhibiting the mycelial growth of isolate CEN216 and this may be one of the mechanisms of control of this pathogen by the antagonist isolates.

Non-volatile metabolites

In the study of the effect of non-volatile metabolites on *S. sclerotiorum*, based on the silhouette method, two inhibition groups were formed, as shown in Table 5. The mean silhouette length was 0.87, which indicates a good separation of the groups of isolates. The best results were achieved with group 2 isolates, namely: CEN1070, CEN1071, CEN1072, CEN1073, CEN1074, CEN1075, CEN1076, CEN1077, CEN1078, CEN1079, CEN1080, CEN129, CEN155, CEN162, CEN201, CEN209, CEN219, CEN273, CEN280, CEN281, CEN287 and CEN289. On the other hand, the results obtained with the other isolates were equal to the control and were allocated to group 2, that is, they did not produce soluble metabolites with properties inhibitory to the pathogen *S. sclerotiorum*.

As for the effect of non-volatile metabolites on *S. rolfsii*, two inhibition groups were formed, as shown in Table 5. The mean silhouette length was 0.8, which indicates a good separation. Composing group 2, with the best results, the isolates appear: CEN1070, CEN1077, CEN1072, CEN1074, CEN1073, CEN1079, CEN1080, CEN1076, CEN1078, CEN1071 and CEN129. These metabolites caused reduced growth of colonies, which were less dense and with deformations. The other isolates make up group 1 and did not produce soluble metabolites with inhibitory action on the mycelial growth of the pathogen.

Suppression of tomato seedlings damping-off in greenhouse

The *Trichoderma* isolates showed to be very promising when evaluated regarding their control of damping-off of tomato seedlings, in greenhouse, with emphasis on the isolates of group 1: CEN126, CEN1070, CEN1080 and CEN281, according to the average silhouette length method (Table 2). These isolates also showed good results in non-volatile metabolites production tests.

Table 4 Grouping of *Trichoderma* isolates regarding the production of volatile metabolites in terms of percentage of inhibition of the growth of the pathogen *Sclerotium rolfsii* (CEN216), based on the mean silhouette length method

Strain	Identification	Group	Diameter of the pathogen colony (cm)	Silhouette Validation
CEN126	*Trichoderma* sp.	3	4.25 ± 0.65	0.75
CEN155	*Trichoderma* sp.	3	4.25 ± 0.65	0.75
CEN170	*Trichoderma* sp.	3	4.25 ± 0.65	0.75
CEN169	*Trichoderma* sp.	3	4.25 ± 0.65	0.75
CEN129	*Trichoderma* sp.	3	4.25 ± 0.65	0.75
CEN290	*Trichoderma* sp.	3	4.25 ± 0.65	0.75
CEN219	*T. afroharzianum*	3	4.25 ± 0.65	0.75
CEN1075	*T. asperellum*	3	4.25 ± 0.65	0.75
CEN273	*Trichoderma* sp.	3	4.25 ± 0.65	0.75
CEN141	*T. afarasin*	1	5.25 ± 0.76	0.52
CEN201	*Trichoderma* sp.	1	5.25 ± 0.76	0.52
CEN161	*Trichoderma* sp.	1	5.25 ± 0.76	0.52
CEN1074	*Trichoderma* sp.	1	5.25 ± 0.76	0.52
CEN289	*Trichoderma* sp.	1	5.25 ± 0.76	0.52
CEN1069	*Trichoderma* sp.	1	5.25 ± 0.76	0.52
CEN288	*Trichoderma* sp.	1	5.25 ± 0.76	0.52
CEN209	*Trichoderma* sp.	1	5.25 ± 0.76	0.52
CEN281	*T. afroharzianum*	1	5.25 ± 0.76	0.52
CEN194	*Trichoderma* sp.	1	5.25 ± 0.76	0.52
CEN1068	*Trichoderma* sp.	1	5.25 ± 0.76	0.52
CEN1073	*Trichoderma* sp.	1	5.25 ± 0.76	0.52
CEN1079	*Trichoderma* sp.	1	5.25 ± 0.76	0.52
CEN162	*T. asperelloides*	1	5.25 ± 0.76	0.52
CEN1072	*Trichoderma* sp.	2	6.29 ± 0.72	0.61
CEN1076	*Trichoderma* sp.	2	6.29 ± 0.72	0.61
CEN316	*Trichoderma* sp.	2	6.29 ± 0.72	0.61
CEN223	*Trichoderma* sp.	2	6.29 ± 0.72	0.61
CEN1078	*Trichoderma* sp.	2	6.29 ± 0.72	0.61
CEN1080	*Trichoderma* sp.	2	6.29 ± 0.72	0.61
CEN1071	*T. brevicompactum*	2	6.29 ± 0.72	0.61
CEN287	*T. afroharzianum*	2	6.29 ± 0.72	0.61
CEN1077	*Trichoderma* sp.	2	6.29 ± 0.72	0.61
CEN1070	*Trichoderma* sp.	2	6.29 ± 0.72	0.61
CEN280	*Trichoderma* sp.	2	6.29 ± 0.72	0.61
Control	-	4	9.00 ± 0.00	0.00

Discussion

Based on the present study, fungi isolate with good antagonistic capacity to the phytopathogenic fungi *S. sclerotiorum* and *S. rolfsii* were selected in laboratory and greenhouse evaluations. Isolates CEN281 and CEN287, both belonging to the species *T. afroharzianum* were the ones that exhibited antagonism in these tests.

As in the present work, some studies describe *Trichoderma* spp. inhibiting in vitro mycelial growth of *S. sclerotiorum* and *S. rolfsii*. Other team members of this work also observed good levels of *S. sclerotiorum* inhibition in dual culture with different *Trichoderma* isolates (Marques et al. 2016, 2022b; Mesquita et al. 2017). Sumida et al. (2018) reported that *T. asperelloides* isolates exhibited inhibition ranging from 60 to 100% of *S. sclerotiorum*. The same was observed in a study by Akhtar and Javaid (2018), where *T. harzianum* inhibited the growth of *S. rolfsii*, with a significant difference when compared to *T. pseudokoninjii* and *T. ressei*. Studies carried out by Kamel et al. (2020) showed that a *T. koningii* isolate exhibited a high inhibitory effect on *S. rolfsii*. Therefore, the interaction *Trichoderma* x soilborne fungi is variable with the species and isolated from the biocontrol agent and also with the phytopathogenic species.

In the dual culture, antagonistic interactions were observed, typically due to competition and hyperparasitism, which according to Papavizas (1985) evidences the interaction between these microorganisms. The observed

Table 5 Grouping of *Trichoderma* isolates regarding inhibition of the growth of the pathogen *Sclerotinia sclerotiorum* (CEN217) by non-volatile metabolites, based on the mean silhouette length method

Strain	*Sclerotinia sclerotiorum*			*Sclerotium rolfsii*		
	Group	Diameter of the pathogen colony (cm)	Silhouette Validation	Group	Diameter of the pathogen colony (cm)	Silhouette Validation
CEN1068	1	8.23 ± 1.19	0.87	1	7.90 ± 1.21	0.77
CEN1069	1	8.23 ± 1.19	0.87	1	7.90 ± 1.21	0.77
CEN1070	2	0.82 ± 0.90	0.87	2	1.95 ± 1.14	0.85
CEN1071	2	0.82 ± 0.90	0.87	2	1.95 ± 1.14	0.85
CEN1072	2	0.82 ± 0.90	0.87	2	1.95 ± 1.14	0.85
CEN1073	2	0.82 ± 0.90	0.87	2	1.95 ± 1.14	0.85
CEN1074	2	0.82 ± 0.90	0.87	2	1.95 ± 1.14	0.85
CEN1075	2	0.82 ± 0.90	0.87	1	7.90 ± 1.21	0.77
CEN1076	2	0.82 ± 0.90	0.87	2	1.95 ± 1.14	0.85
CEN1077	2	0.82 ± 0.90	0.87	2	1.95 ± 1.14	0.85
CEN1078	2	0.82 ± 0.90	0.87	2	1.95 ± 1.14	0.85
CEN1079	2	0.82 ± 0.90	0.87	2	1.95 ± 1.14	0.85
CEN1080	2	0.82 ± 0.90	0.87	2	1.95 ± 1.14	0.85
CEN126	1	8.23 ± 1.19	0.87	1	7.90 ± 1.21	0.77
CEN129	2	0.82 ± 0.90	0.87	2	1.95 ± 1.14	0.85
CEN141	1	8.23 ± 1.19	0.87	1	7.90 ± 1.21	0.77
CEN155	2	0.82 ± 0.90	0.87	1	7.90 ± 1.21	0.77
CEN161	1	8.23 ± 1.19	0.87	1	7.90 ± 1.21	0.77
CEN162	2	0.82 ± 0.90	0.87	1	7.90 ± 1.21	0.77
CEN169	1	8.23 ± 1.19	0.87	1	7.90 ± 1.21	0.77
CEN170	1	8.23 ± 1.19	0.87	1	7.90 ± 1.21	0.77
CEN194	1	8.23 ± 1.19	0.87	1	7.90 ± 1.21	0.77
CEN201	2	0.82 ± 0.90	0.87	1	7.90 ± 1.21	0.77
CEN209	2	0.82 ± 0.90	0.87	1	7.90 ± 1.21	0.77
CEN219	2	0.82 ± 0.90	0.87	1	7.90 ± 1.21	0.77
CEN223	1	8.23 ± 1.19	0.87	1	7.90 ± 1.21	0.77
CEN273	2	0.82 ± 0.90	0.87	1	7.90 ± 1.21	0.77
CEN280	2	0.82 ± 0.90	0.87	1	7.90 ± 1.21	0.77
CEN281	2	0.82 ± 0.90	0.87	1	7.90 ± 1.21	0.77
CEN287	2	0.82 ± 0.90	0.87	1	7.90 ± 1.21	0.77
CEN288	1	8.23 ± 1.19	0.87	1	7.90 ± 1.21	0.77
CEN289	2	0.82 ± 0.90	0.87	1	7.90 ± 1.21	0.77
CEN290	1	8.23 ± 1.19	0.87	1	7.90 ± 1.21	0.77
CEN316	1	8.23 ± 1.19	0.87	1	7.90 ± 1.21	0.77
Control	1	8.23 ± 1.19	0.87	1	7.90 ± 1.21	0.77

alterations evidence the parasitism of *Trichoderma* on the studied pathogens, as suggested by Papavizas (1985). Corroborating what was reported in this work, studies by Troian et al. (2014) and Kamel et al. (2020) showed that *Trichoderma* spp. has high levels of gene expression when parasitizing *S. sclerotiorum* and *S. rolfsii*, in addition to a synergistic action with the production of enzymes that degrade the fungal cell wall. Enzymes produced by *Trichoderma* spp., such as chitinases and cellulases, are known to lyse or degrade the pathogen's cell wall (Ghasemi et al. 2020). Louzada et al. (2009) emphasize that only part of the evaluated *Trichoderma* isolates hyperparasitized *S. sclerotiorum*, suggesting that other mechanisms could be involved in the antagonism in vitro.

The results obtained in this work agree with those obtained by different authors, indicating that fungi of the genus *Trichoderma* have the ability to produce volatile metabolites with an inhibitory effect on the mycelial growth of several other fungi in the laboratory (Dennis and Webster 1971b). Kushwaha et al. (2018) observed an intermediary inhibition of this pathogen by *T. harzianum, T. viride* and *T. virens*. Among the metabolites produced by this group

of fungi, there are gases, such as ethylene and hydrogen cyanide (Campbell 1989), which interfere with microbial growth. These volatile metabolites have activity at low concentrations but are not considered antibiotics. Besides these, the VOCs (Volatile Organic Compounds) acetoin, 2-methyl-1-propanol, 3-methyl-1-butanol and 6-pentyl-2-pyrone were the most ambiguous and produced by *T. atroviride*, when confronted with *Phytophthora infestans* (Elsherbiny et al. 2020).

Some non-volatile compounds produced by *Trichoderma* spp. have already been identified, in the pioneering work of Weidling (1934), where antibiotics such as gliotoxin and viridine were described; subsequentlyDennis and Webster (1971a),b) demonstrated the ability of some *Trichoderma* isolates to produce volatile and non-volatile compounds with an inhibitory effect on the development of several fungi. Among the effects caused by antibiotics, the reduction or stoppage of the growth and sporulation of the phytopathogen, reduction of spore germination, hyphal deformation and hydrolysis deserve to be highlighted (Campbell 1989). More examples of metabolites produced by the antagonist are pyrones, acids, furans, and lipids (Leylaie and Zafari 2018). Studies performed by other team members showed that non-volatile metabolites from *T. ganense* was not thermolabile and was able to completely inhibit *S. sclerotiorum* (Louzada et al. 2016), just as *T. brevicompactum* produced non-volatile metabolites capable of inhibiting the growth of *S. rolfsii* and *S. sclerotiorum*, among other phytopathogenic fungi (Marques et al. 2018). Kushwaha et al. (2018) also found that isolates of *T. harzianum, T. viride* and *T. virens* produced non-volatile metabolites with inhibitory action on *S. rolfsii*. Marques et al. (2022b) observed inhibition of mycelial growth of *S. sclerotiorum* by non-volatile metabolites of *T. afroharzianum* of up to 51.19%.

These data obtained corroborate others existing in the literature. As an example, Islam et al. (2016) reported that the incidence of tomato collar rot caused by *S. rolfsii* was lower in the treatment with *T. harzianum*, when compared with *T. virens* and *T. asperellum*. Later, Suriyagamon et al. (2018) showed satisfactory results in the biocontrol of *S. rolfsii* also in tomato, with isolates of *T. harzianum* and *T. konnigii*, respectively. Also under greenhouse conditions, *T. koningii, T. viride* and *T. harzianum* showed the highest antagonistic effect against three isolates of *S. rolfsii*, according to Kamel et al. (2020), as well as *T. asperellum* reduced the severity of this disease when associated with ammonium nitrate (Blanco et al. 2021).

Thirteen new *Trichoderma* isolates were obtained in areas cultivated with tomato at Embrapa Hortaliças (CNPH), Brazil, and incorporated into the Collection of Fungi for Biological Control of Embrapa/CENARGEN, expanding its gene pool. A variation in in vitro antagonism was observed both between species and within species of the evaluated antagonist fungi. According to these tests, isolates CEN281 and CEN287, both belonging to the species *T. afroharzianum*, showed potential as biocontrol agents for the phytopathogenic fungi *Sclerotinia sclerotiorum* and *Sclerotium rolfsii*. The isolates studied do not have inhibitory action against *S. sclerotiorum* due to the production of volatile metabolites. However, it is concluded that all of them were able to inhibit the growth of *S. rolfsii* by the production of these compounds. Isolates CEN281, CEN1070 and CEN1080 should be used in field studies, as they have presented the best results in the control of damping-off of tomato seedlings, caused by *S. rolfsii*, in greenhouse.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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