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Research Article
Mycelial Inhibition of *Sclerotinia sclerotiorum* by *Trichoderma* spp. Volatile Organic Compounds in Distinct Stages of Development

[1]Lincon Rafael da Silva, [2]Paulo Henrique Pereira Costa Muniz, [1]Gustavo Henrique Silva Peixoto, [1]Bruna Eliza Gonçalves Dias Luccas, [3]João Batista Tavares da Silva and [3]Sueli Corrêa Marques de Mello

[1]Department of Phytopathology, University of Brasilia, Brasilia, Federal District, Brazil
[2]Department of Phytopathology, State University of Goiás, Ipameri, Goiás, Brazil
[3]Embrapa Genetic Resources and Biotechnology, Brasilia, Federal District, Brazil

Abstract

Background and Objective: Fungi of the genus *Trichoderma* have high versatility in the control of different plant diseases. Among the main mechanisms of action of these fungi against phytopathogenic fungi, the production of Volatile Organic Compounds (VOCs) is mentioned. These compounds are said to inhibit the mycelial growth of various fungal pathogens. The objective of this work was to evaluate the *in vitro* inhibition of the mycelial growth of *Sclerotinia sclerotiorum* by VOCs from six *Trichoderma* strains in different stages of development of the biocontrol agent. **Materials and Methods:** In this work, the *in vitro* evaluation of the mycelial growth of the phytopathogen *S. sclerotiorum* by VOCs from six *Trichoderma* strains was carried out: *T. koningiopsis* (CEN1386), *T. asperelloides* (CEN1397), *T. longibrachiatum* (CEN1399) *T. lentiforme* (CEN1416), *T. perbedyi* (CEN1389) and *T. azevedoi* (CEN1241). Observations were made at different stages of antagonist development: mycelial Growth Phase (GP), Sporulation Phase (SP) and paired with the Pathogen Phase (PP). Besides, the sporulation of the tested strains was quantified. **Conclusion:** In all experimental conditions, the VOCs produced by the CEN1241 strain showed a greater inhibitory effect, although the inhibition was less evident when the cultures of *S. sclerotiorum* were exposed in the GP phase of the antagonist. Greater sporulation was observed with *T. lentiforme* (CEN1416), a fact not related to a better ability to inhibit *S. sclerotiorum*, by VOCs.

Key words: Antagonism, biological control, secondary metabolites, white mould, sporulation, dual culture, plant disease

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Corresponding Author: Sueli Corrêa Marques de Mello, Embrapa Genetic Resources and Biotechnology, Parque Estação Biologica W5 Norte, Asa Norte, 70770-900, Brasilia, Federal District, Brazil Tel: +55-61-3448-4776

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The fungus *Sclerotinia sclerotiorum* (Lib) de Bary has been reported to cause the disease known worldwide as white mould, in several crops[1]. Potential epidemics can develop from the germination, both myceliogenic and carpogenic, of survival structures (sclerotia), present in the soil or introduced with the seeds[2].

The chemical control of white mould depends on discovering the symptoms quickly, to prevent the formation of sclerotia and, the subsequent spread of the disease in the field. However, the use of chemical fungicides has led to the selection of resistant populations of the pathogen, a fact that compromises the efficiency of this control method[3]. Biological control is seen as an option and may be combined with synthetic fungicides[4,5].

Soil fungi of the genus *Trichoderma* Persoon have the ability to establish a beneficial symbiosis with plants[6]. These fungi have been attracting interest for studies in basic and applied mycology because several of their species have potential as agents of biological control[7]. The production of volatile organic compounds is one of the modes of action by which *Trichoderma* spp. act against phytopathogens[8].

Volatile Organic Compounds (VOCs) are hydrophobic substances of low molecular weight, which can evaporate in ambient temperature and pressure conditions. These substances may play an important role in long-distance communication between organisms[9]. These compounds belong to different chemical classes, including monoterpenes, sesquiterpenes, alcohols, ketones, lactones and esters, among others[10]. Several studies show that certain VOCs produced by *Trichoderma* spp. can inhibit the mycelial growth of phytopathogenic fungi[5,11,12].

The production of VOCs by *Trichoderma* is quite diversified both qualitatively and quantitatively[13]. This diversity is related to abiotic factors such as temperature, humidity and substrate and biotic factors, such as the species and age of the colony. As it is a living organism, when *Trichoderma* is applied in the field, it requires time to establish itself in the environment to then exercise biocontrol action. It is reasonable to think that the inhibition of mycelial growth of phytopathogens by *Trichoderma* VOCs becomes more effective after the establishment and full functioning of the antagonist's metabolism.

Thus, the objective of this work was to evaluate, *in vitro*, the inhibition of the mycelial growth of *S. sclerotiorum* by VOCs from six *Trichoderma* strains in different stages of development of the biocontrol agent.

MATERIALS AND METHODS

Study area: The study was carried out at Embrapa Genetic Resources and Biotechnology, Laboratory Phytopathology, Brasilia, Federal District, Brazil from August-December, 2019.

Origin and maintenance of strains: *Trichoderma* strains belonging to the species *T. koningiopsis* (CEN1386), *T. asperelloides* (CEN1397), *T. longibrachiatum* (CEN1399), *T. lentiforme* (CEN1416), *T. perbedyi* (CEN1389) and *T. azevedoi* (CEN1241) were evaluated against the pathogen *S. sclerotiorum* (CEN1147). All of these fungi belong to the Collection of Biological Control Agents for Phytopathogens and Weeds of Embrapa Genetic Resources and Biotechnology, Brasilia, Federal District, Brazil and were maintained in liquid nitrogen. The cultures were reactivated in Potato Dextrose Agar (PDA) medium, seeded into test tubes with screw cap, containing 20 mL of the same medium and stored in a refrigerator at 6°C, for later use.

Mycelial inhibition of *S. sclerotiorum* after exposure to VOCs emitted by *Trichoderma* spp. in the mycelial Growth Phase (GP): Petri dishes (150×25 mm) containing 60 mL solidified PDA medium, received one PDA disk (5 mm Ø) colonized by *Trichoderma* in the centre. In the same way, in another Petri dish, a disk of PDA (5 mm Ø) colonized by the pathogen was placed. The distance between cultures was approximately 28.6 mm. The dishes containing antagonists and pathogens were superimposed to form a shared atmosphere. The set was sealed with plastic paraffin film and wrapped with transparent plastic so that the bases containing the pathogen were in the upper position. The control treatment was represented by Petri dishes with *S. sclerotiorum*, but without the antagonist at the bottom. The cultures were incubated at a temperature of 25°C, with a 12 hrs photoperiod. Daily, colony diameter measurements were taken with a digital calliper (Digimess Precision Instruments®, Sao Paulo, Brazil) until total colonization of the PDA in control treatments. The average diameter values of the colonies were used for the calculation of the Area Under the Mycelial Growth Curve (AUMGC):

$$AUMGC = \sum\left[\frac{y1+y2}{2}\times T2\text{-}T1\right]$$

where, y1 and y2 are two consecutive evaluations performed at times T1 and T2, respectively.

Each treatment was performed in triplicate. The experiment was repeated to confirm the results.

Mycelial inhibition of *S. sclerotiorum* after exposure to VOCs emitted by *Trichoderma* spp. in Sporulation Phase (SP): Petri dishes (150×25 mm) containing 60 mL solidified PDA medium received, in the centre, one PDA disk (5 mm Ø) colonized by *Trichoderma*. After 96 hrs, Petri dishes of a corresponding size containing 60 mL of solidified PDA, received a disc of PDA (5 mm Ø) colonized by the pathogen. The distance between cultures was approximately 28.6 mm. Dishes containing the antagonist in the SP were superimposed with plates containing the pathogen, forming a shared atmosphere. The control treatment was represented by Petri dishes with *S. sclerotiorum*, but without the antagonist at the bottom. The cultures were incubated in the same conditions as described above. The evaluations were carried out according to the previous item. The experiment was repeated to confirm the results.

Mycelial inhibition of *S. sclerotiorum* after exposure to VOCs emitted by *Trichoderma* spp. Paired with the Pathogen (PP): Petri dishes (150×25 mm) containing 60 mL of solidified PDA medium received, at one end, one PDA disc (5 mm Ø) colonized by *Trichoderma*. Similarly, the pathogen was positioned on the side opposite the antagonist. The cultures were incubated at 25°C, with a 12 hrs photoperiod. After 96 hrs, plates of corresponding size containing 60 mL of solidified PDA, received, in its centre, a disk of PDA (5mm Ø) colonized by the pathogen and were placed overlapping the plates containing the culture pairing, forming a shared atmosphere. The distance between cultures was approximately 28.6 mm. The control treatment was represented by Petri dishes with *S. sclerotiorum*, but with only PDA at the bottom. These cultures were incubated in the same conditions as described above. The evaluations were carried out as described in the previous section. The experiment was repeated to confirm the results.

Quantification of spores produced by strains of *Trichoderma*: To quantify the spores, 20 mL of Sterile Distilled Water (SDW) was added to each Petri dish, followed by the release of the spores with a Drigalski handle. Then the spores were collected in beakers and filtered through sterile gauze (Descarpack®, Joinville, SC, Brazil). The spore suspensions were examined was examined under a microscope with the aid of a Neubauer chamber and the number of spores determined by counts (five samples/plate). The experiment was repeated to confirm the results.

Statistical analysis: The data were submitted to analysis of variance, followed by the application of the Scott-Knott test to compare the means ($p<0.05$). Regression analysis was also performed to obtain significant models. In all analyses, the statistical program used was Sisvar 5.3[14].

RESULTS

The VOCs produced by *Trichoderma* strains used in this study inhibited the *S. sclerotiorum* colonies growth, in the three experimental conditions. The mycelial growth of *S. sclerotiorum* exposed and unexposed to *Trichoderma* spp. was adjusted to quadratic regression models with R^2 greater than 0.98 (Fig. 1a-f).

When pathogen and antagonist were exposed simultaneously, the mycelial growth rate of the pathogen was inhibited to the control, after 72 hrs of exposure in most tested strains. The *T. azevedoi* CEN1241 was the one that showed the greatest inhibitory effect on mycelial growth (Fig. 1e).

When exposed to VOCs produced by *Trichoderma* spp. colonies in the sporulation phase, there was an increase in the inhibition of the mycelial growth of *S. sclerotiorum*, in comparison to the cultures inoculated simultaneously (GP). Mycelial growth was adjusted to quadratic regression models (Fig. 2a-f). All strains showed the ability to inhibit the mycelial growth of the pathogen, in the initial moment of cultivation, when compared to the control.

The *Trichoderma* strains with the highest inhibition rates were *T. azevedoi* CEN1241, *T. koningiopsis* CEN1386 and *T. asperelloides* CEN1397. These showed rates of mycelial growth inhibition of the pathogen greater than 80% (Fig. 3a-g), indicating that these strains acted directly in inhibiting the growth of the pathogen, by the action of VOCs.

To exposure in PP condition, presented the highest growth inhibition rates of *S. sclerotiorum.* Again, mycelial growth was adjusted to quadratic regression models (Fig. 4a-f).

A treatment containing only the pathogen at both ends of the shared atmosphere was inserted in the PP assay (Fig. 5a-h), demonstrating that the diameters of the pathogen

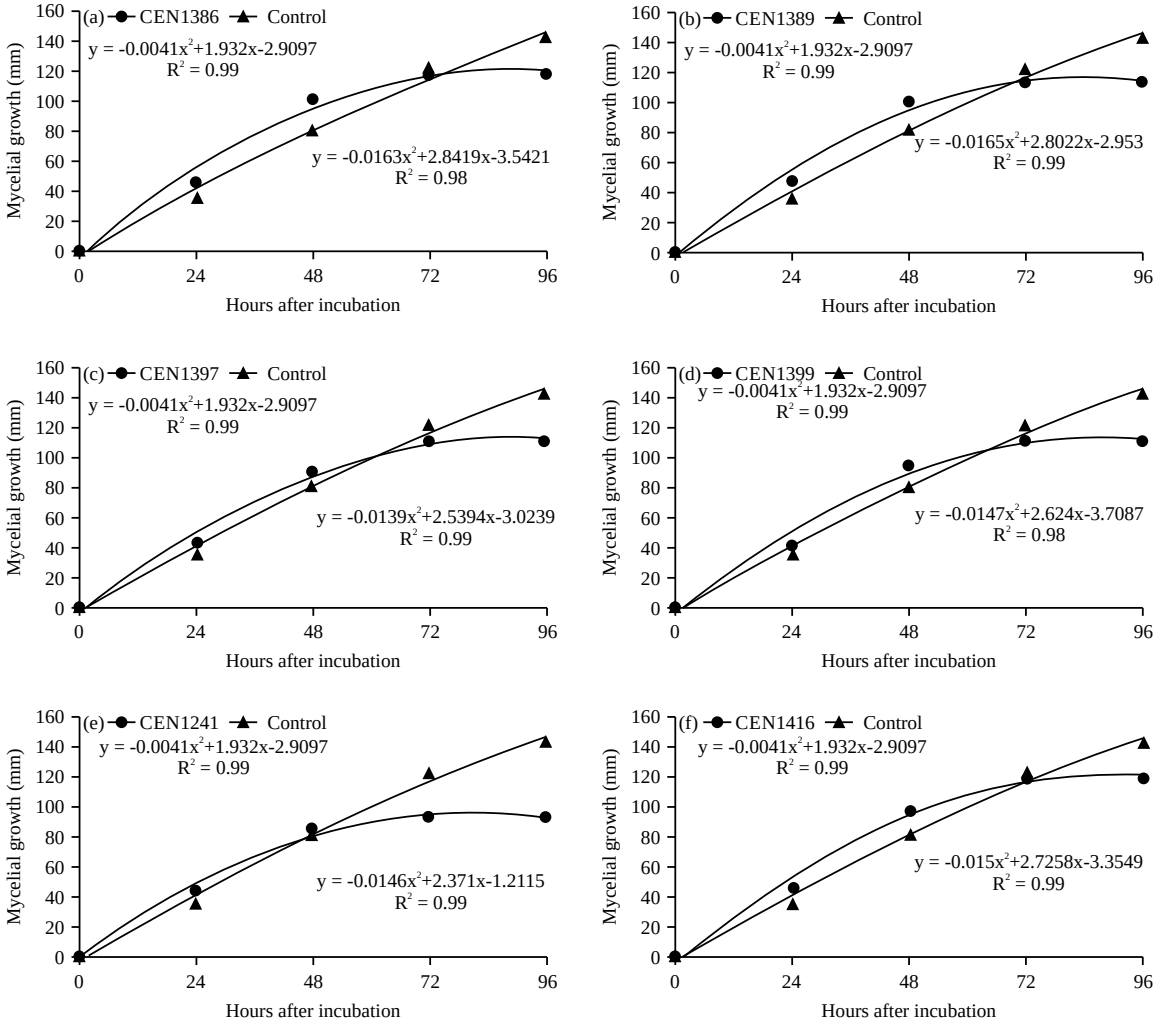

Fig. 1(a-f): Mycelial growth of *S. sclerotiorum* exposed to VOCs of *Trichoderma* spp. in the mycelial growth phase during 96 hrs of cultivation on PDA medium

(a) *T. koningiopsis* CEN1386, (b) *T. perbedyi* CEN1389, (c) *T. asperelloides* CEN1397, (d) *T. longibrachiatum* CEN1399, (e) *T. azevedoi* CEN1241 and (f) *T. lentiforme* CEN1416

colonies were similar (Fig. 5h). However, the colonies of this treatment had a smaller diameter compared to the control treatment, only on PDA medium (Fig. 5g). Based on this observation, it is inferred that there is a slight competition for oxygen within the shared atmosphere. Thus, the mycelial growth inhibitory mechanism of the pathogen could not only be due to the antibiosis mechanism but also due to competition for oxygen. Future studies should be developed to better assess this finding.

Regarding the statistical results, there was significant interaction for *Trichoderma* strains and tested exposure conditions, when comparing data from the area under the pathogen mycelial growth curve (AUPMG). *T. azevedoi* CEN1241 was the strain that exhibited the best antifungal action, providing the lowest values of AUPMG in all conditions of exposure to the pathogen (Table 1). The other strains showed inhibitory activity with lower AUPMG values than the control, except for the development stage (GP).

Regarding exposure conditions, culture pairing showed the highest mycelial growth inhibition of *S. sclerotiorum*, whose mean values were statistically higher than the other conditions evaluated (Table 1). Concerning the number of spores produced by *Trichoderma* spp., strain CEN1416 was statistically superior to the others tested (Table 1).

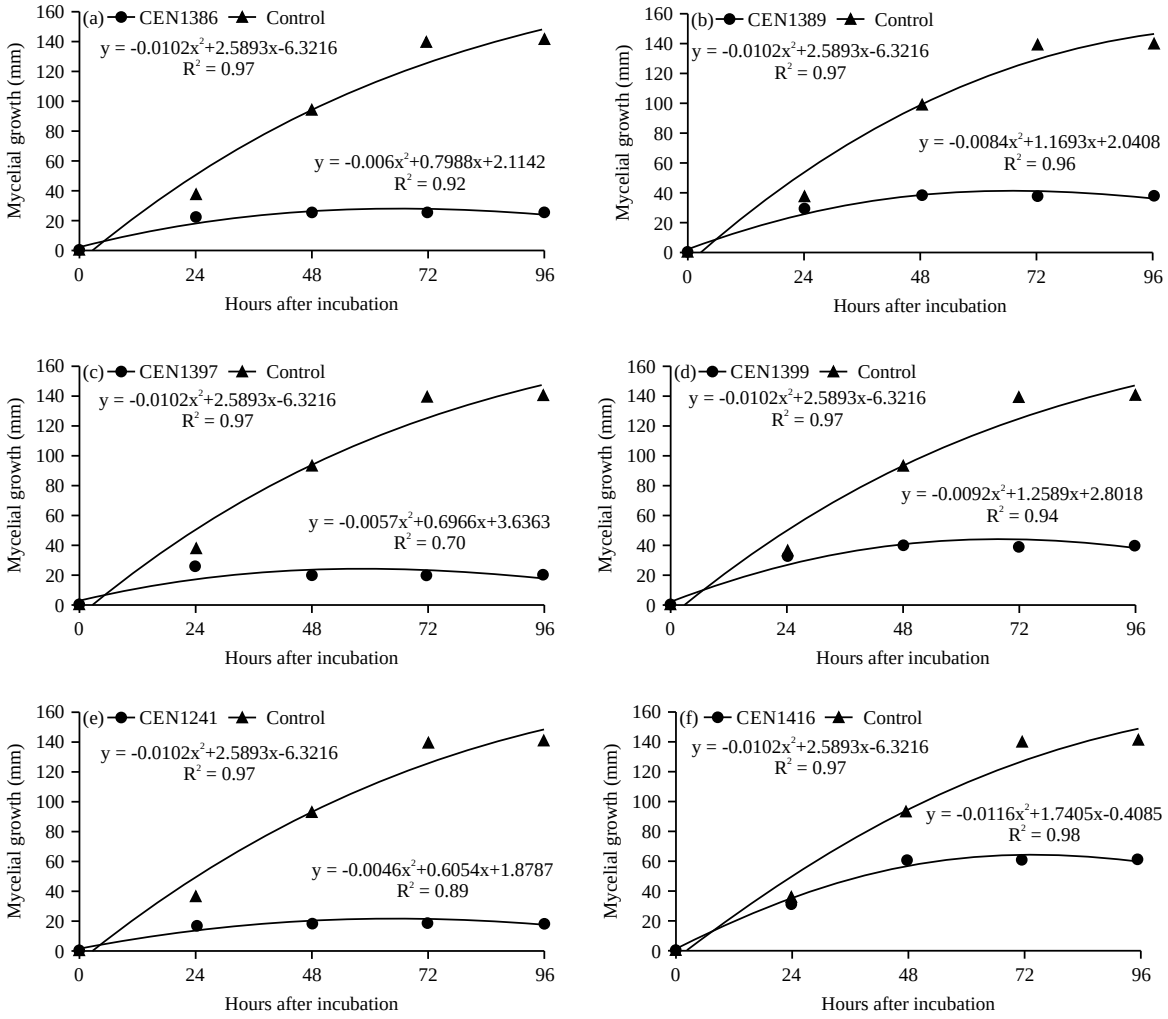

Fig. 2(a-f): Mycelial growth of *S. sclerotiorum* exposed to VOCs of *Trichoderma* spp. in the sporulation phase during 96 hrs of cultivation on PDA medium

(a) *T. koningiopsis* CEN1386, (b) *T. perbedyi* CEN1389, (c) *T. asperelloides* CEN1397, (d) *T. longibrachiatum* CEN1399, (e) *T. azevedoi* CEN1241 and (f) *T. lentiforme* CEN1416

Table 1: Mean values of Area Under the Pathogen Mycelial Growth Curve (AUPMG) of *S. sclerotiorum* exposed to *Trichoderma* spp.

	AUPMG (mm^2)[1,2]			Sporulation
Strains	GP[2]	SP[2]	PP[2]	($\times 10^8$ conidia mL^{-1})[1]
CEN1241	5829.33^{aB}	1362.33^{aA}	1465.70^{aA}	3.98±0.12^{d}
CEN1386	7229.93^{cB}	1806.23^{aA}	1893.63^{bA}	3.93±0.35^{d}
CEN1389	7012.23^{cB}	2668.46^{bA}	2625.00^{cA}	0.30±0.04^{e}
CEN1397	6654.36^{bB}	1872.46^{aA}	1676.20^{aA}	5.93±0.85^{c}
CEN1399	6739.53^{cB}	2858.83^{bB}	1994.76^{bA}	8.83±0.45^{b}
CEN1416	7110.73^{cC}	4052.60^{cB}	2493.83^{cA}	12.01±1.01^{a}
Control	6958.20^{cA}	7785.60^{dB}	7508.23^{dB}	-
Mean	6790.61^{C}	3200.93^{B}	2808.05^{A}	-
CV (%)	2.59	9.52	7.05	11.07

Means followed by the same superscripted lower-case letters in the columns and upper-case letters in the lines do not differ statistically, according to the Scott-Knott test ($p \leq 0.05$). VOCs in different stages of development: mycelial Growth Phase (GP), Sporulation Phase (SP), pairing with the pathogen (PP) and sporulation of *Trichoderma* strains and Area Under the Pathogen Mycelial Growth (AUPMG)

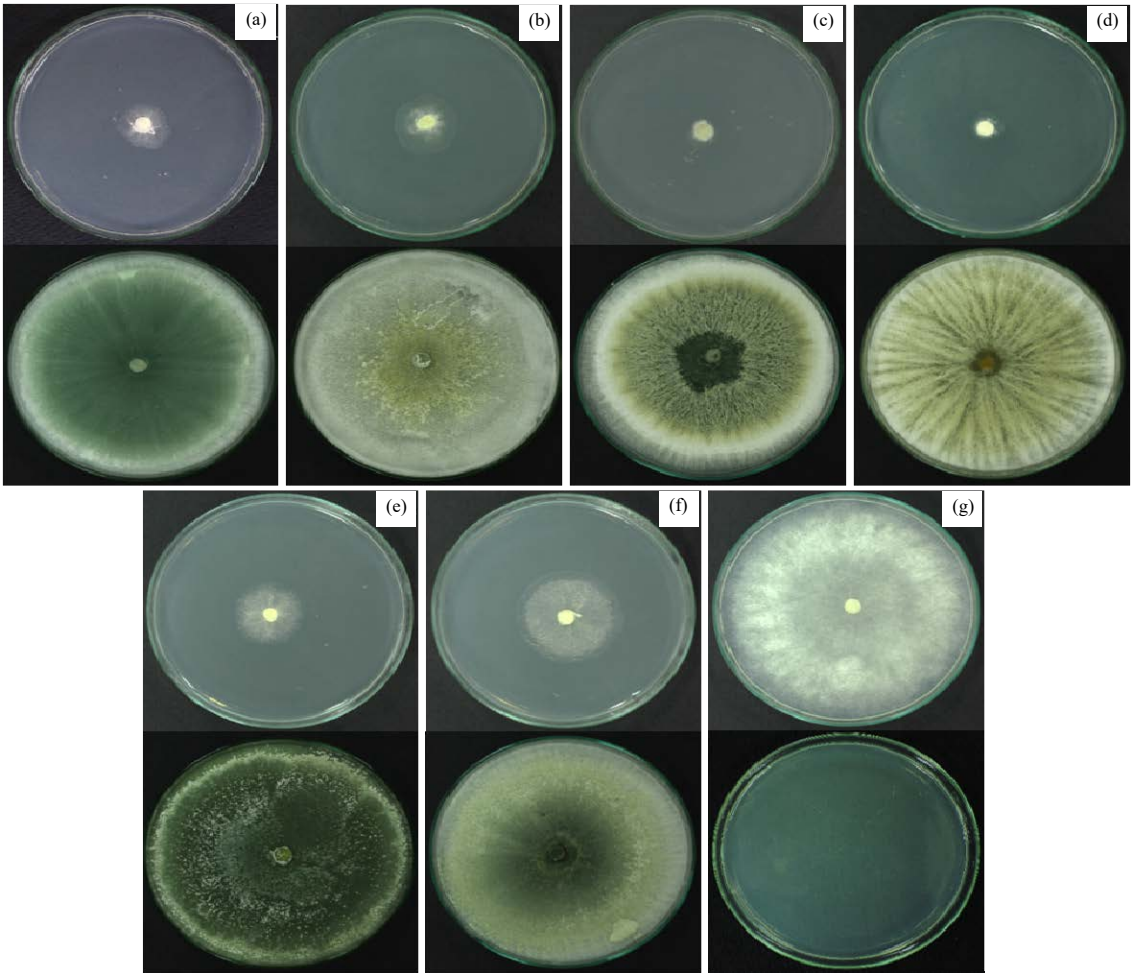

Fig. 3(a-g): Mycelial growth inhibition of *S. sclerotiorum* exposed to *Trichoderma* spp. VOCs. in the sporulation phase on PDA medium

(a) *T. koningiopsis* CEN1386, (b) *T. perbedyi* CEN1389, (c) *T. asperelloides* CEN1397, (d) *T. azevedoi* CEN1241, (e) *T. longibrachiatum* CEN1399, (f) *T. lentiforme* CEN1416 and (g) Control

DISCUSSION

In the end, the results showed that there are divergences between the conditions tested. It was possible to identify a greater mycelial inhibition of *S. sclerotiorum* CEN1147 when exposed to the condition of culture paired with the pathogen (PP) and in sporulation (SP) to a simultaneous condition of growth of antagonist and pathogen (GP). Regardless of the condition tested, *T. azevedoi* CEN1241 was the one that showed the greatest inhibitory effect on mycelial growth *S. sclerotiorum* CEN1147. It is still worth noting that the greater potential for mycelial inhibition of the pathogen was not related to the greater ability to produce spores of *T. lentiforme* CEN1416.

In simultaneous growth conditions of the antagonist and pathogen, our results are following the study by Silva *et al.*[15] who reported inhibition averages between 15 and 35.2%, using different species of *Trichoderma*. Ojaghian *et al.*[5], using VOCs from 11 species of *Trichoderma*, demonstrated that these compounds produced an inhibitory effect of 10-80% of the mycelial growth of *S. sclerotiorum*. Antibiosis exerted by VOCs is an important action mechanism of *Trichoderma* and has been used as one of the parameters for the selection of strains of these fungi as biocontrol agents[16,17]. Phytopathogen-inhibiting VOCs are derived from different pathways of fungal metabolism and consist of a wide range of compounds, including pyrones, terpenes, alcohols, ketones, xanthones, alkanes, alkenes and phenols[15,16,18].

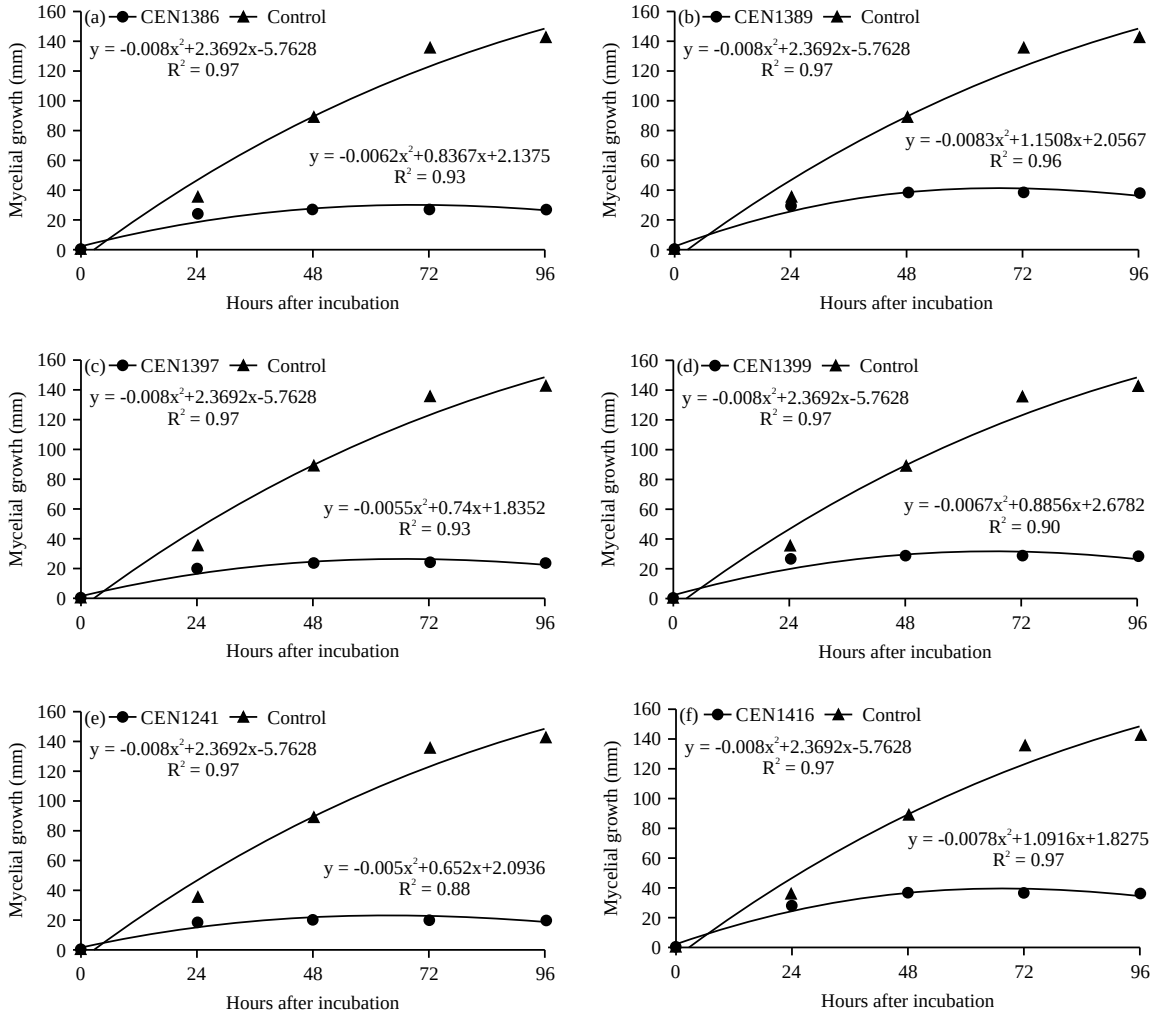

Fig. 4(a-f): Mycelial growth of *S. sclerotiorum* exposed to *Trichoderma* spp. COVs. in pairing methodology with the pathogen during 96 hrs of cultivation on PDA medium

(a) *T. koningiopsis* CEN1386, (b) *T. perbedyi* CEN1389, (c) *T. asperelloides* CEN1397, (d) *T. longibrachiatum* CEN1399, (e) *T. azevedoi* CEN1241 and (f) *T. lentiforme* CEN1416

Unlike synthetic fungicides that, after application in the field, can already act on pathogens, biological fungicides require time to establish themselves in the soil (vegetative and reproductive stages), before starting their activity, due to their different mechanisms of action against pathogens[8,19,20]. The transition from vegetative growth to the conidial stage in many fungi is marked by an increase in the production of secondary metabolites[21]. This was the key point of observation for our research, regarding the sporulation stage of *Trichoderma*.

Some pathogens, such as *S. sclerotiorum*, have rapid vegetative growth[22]. This can lead to unsatisfactory results for the biocontrol agent, in evaluations with conditions of simultaneous cultivation with the pathogen. In this work, it was observed that when the pathogen is incubated in the presence of *Trichoderma* in the sporulation stage, its mycelial growth is more efficiently inhibited (Fig. 2). This may explain the good results found in the literature, obtained with preventive applications of the biocontrol agent, for example, in the form of seed treatment[4,23,24].

Until the execution of this work, no results were found in the literature obtained with the culture methodology paired with the pathogen. So, this is the first study using this approach. From the results obtained in the PP tests, it appears that *Trichoderma*, when subjected to stress from antagonism against the pathogen, could alter its VOC emission

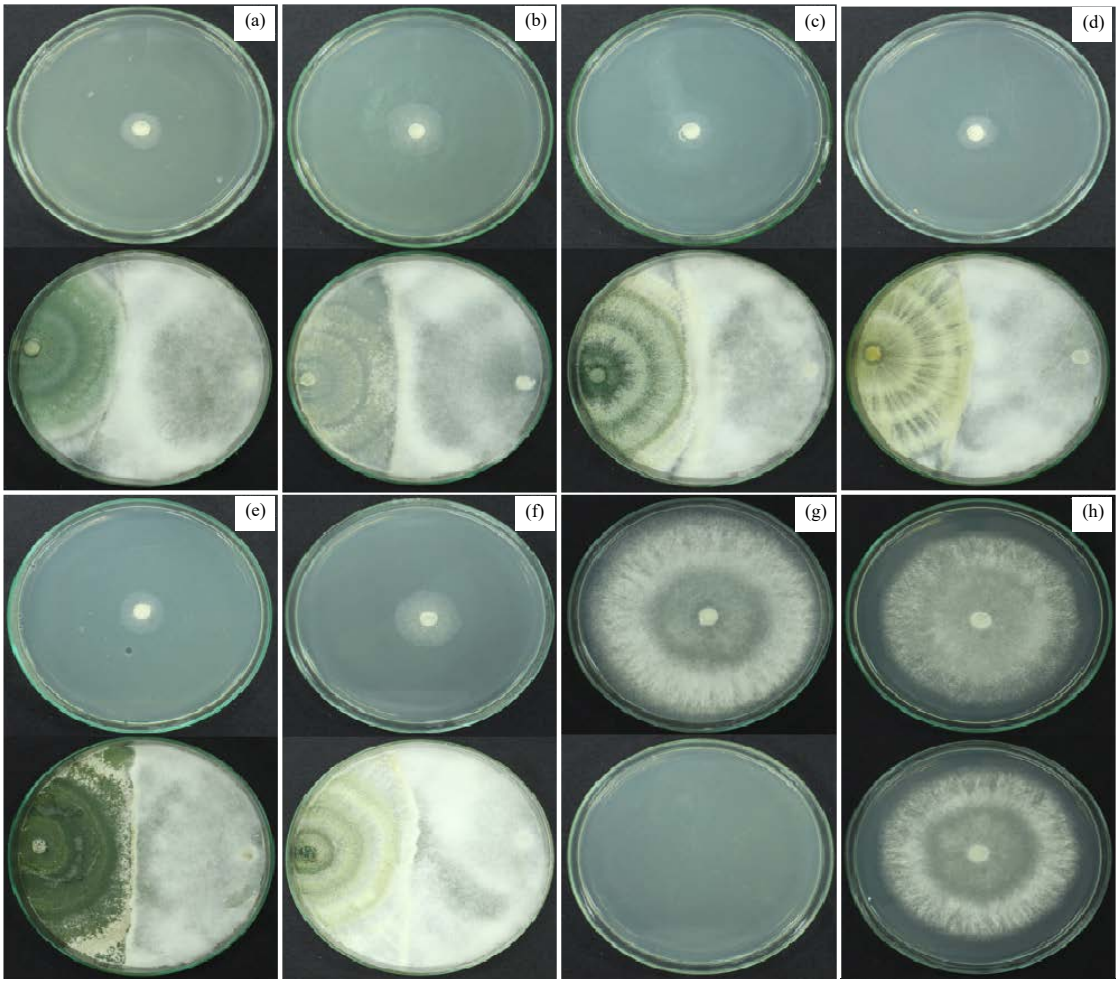

Fig. 5(a-h): Mycelial growth inhibition of *S. sclerotiorum* exposed to *Trichoderma* spp. VOCs in pairing methodology with the pathogen on PDA medium

(a) *T. koningiopsis* CEN1386, (b) *T. perbedyi* CEN1389, (c) *T. asperelloides* CEN1397, (d) *T. azevedoi* CEN1241, (e) *T. longibrachiatum* CEN1399, (f) *T. lentiforme* CEN1416, (g) Control (*S. sclerotiorum*+PDA) and (h) Control (*S. sclerotiorum* × *S. sclerotiorum*)

profile, enhancing its ability to inhibit mycelial growth in *S. sclerotiorum* by the antibiosis mechanism. VOCs in this experimental condition were able to markedly reduce the mycelial growth of the pathogen (Fig. 5). The VOC profile is characteristic for each fungus species; however, it can be changed by the nutritional conditions of the growing substrate, age of the culture and during antagonistic interaction with other fungi[25,26]. In a study by Guo *et al.*[13], an increase in the total emission of VOCs from *T. hamatum* was found in confrontation with *Laccaria bicolor*, with 19 new VOCs being identified. On the other hand, there was a reduction in the emission of VOCs by *T. harzianum*. Mutawila *et al.*[20], showed that VOC 6-pentyl- α-pyrone (6PAP) increased significantly in *T. atroviride* in Co-culture with the pathogen *Neofusicoccum parvum*. 6PAP is a well-known antimicrobial compound produced by species of *Trichoderma*[27-29].

In all experimental conditions used in the present work, the lowest values of AUPMG were obtained by *T. azvedoi* CEN1241. Similar to this study, other studies have also shown that the percentage of mycelial inhibition of phytopathogens by *Trichoderma* VOCs can be very diverse among the species of the biocontrol agent[5,30]. This may be related to the VOC profile emitted by each *Trichoderma* species/strain[13]. The atmosphere shared between the fungi, formed from the inverted plate methodology, can induce specific signals of communication and defence between them, which can result in the diversified emissions of the biocontrol agent VOCs in each condition[13]. These results indicate the complexity of the events that occur during biological control by *Trichoderma*.

Regardless of the strain, special attention should be paid to the period in which the effective onset of mycelial inhibition is observed in the non-sporulating condition. The data of Fig. 1 shows that the effective mycelial inhibition rates of *S. sclerotiorum* begin to increase after 72 hrs of *Trichoderma* growth. Jelén *et al.*[29] showed that *T. atroviride* significantly increases the production of different VOCs such as 2-heptanone, 2-nonanone, 1-octene-3-ol and 6PAP, after approximately 96 hrs of growth in PDA medium. This provides evidence of the real need for the colony to be in full development to obtain better results for pathogen inhibition by *Trichoderma* VOCs.

At the end of the test in the sporulation condition, we tried to relate the best potential for mycelial inhibition with the number of spores produced, relative to each of the *Trichoderma* strains. However, the strain CEN1416, which has been shown to produce the largest number of conidia, was the least effective in inhibiting the mycelial growth of *S. sclerotiorum*. A study by Nemcovic *et al.*[21] reported an increase in the production of VOCs of eight carbons (1-octen-3-ol, 3-octanol and 3-octanone) by conidial colonies of *T. atroviride*. These authors suggest that the production of some VOCs is linked to the process of forming conidia. However, in this study, this relationship was not investigated. As previously mentioned, mycelial inhibition of the pathogen seems to be more related to the qualitative profile of VOCs emitted by *Trichoderma* spp. It is worth noting that strains that have good sporulation capacity, even without effective VOC inhibitors, can also be selected as biocontrol agents, as they will act by another mechanism of action, such as hyperparasitism.

CONCLUSION

In all experimental conditions, VOCs produced by *T. azevedoi* CEN1241 statistically exceeded those of the other strains, concerning the mycelial inhibition of *S. sclerotiorum* CEN1147. The mycelial inhibition of *S. sclerotiorum* CEN1147 by VOCs produced by *Trichoderma* spp. was more evident when using the SP and PP methodologies. The greater sporulation capacity of *T. lentiforme* CEN1416 is not related to the greater ability to inhibit the mycelial growth of *S. sclerotiorum* CEN1147 by the production of VOCs.

SIGNIFICANCE STATEMENT

Several studies were conducted *in vitro* with the antagonist fungus *Trichoderma* spp. when interacting with phytopathogens, such as *S. sclerotiorum*, always bring the same mycelial inhibition methodology, especially when the antibiosis mechanism is used through the emission of Volatile Organic Compounds (VOCs). In this work, we brought unpublished results on new methodologies for evaluating the interaction of *Trichoderma* spp. and *S. sclerotiorum*. We showed that these *in vitro* results may help future field trials, as well as the tested methodologies, show that *Trichoderma* spp. has greater control efficiency when it is provided with stabilization and growth time so that its entire VOC arsenal is available to inhibit the phytopathogen.

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