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Original Article

Silviculture

Antagonism and Effect of Volatile Metabolites of *Trichoderma* spp. on *Cladosporium* spp.

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ABSTRACT

Pecan is susceptible to the occurrence of diseases capable of harming its development, and leaf spot caused by *Cladosporium* spp is among them. Therefore, the objective of this work was to analyze the potential antagonist and *in vitro* effect of volatile metabolites of *Trichoderma*spp. On *Cladosporium* spp. isolated from pecan leaf spots. Three *Cladosporium* spp. isolates and two commercial products based on *Trichoderma*spp. were used, as well as an isolate of the same genus provided by the 'ElocyMinussi' Laboratory of Phytopathology at *UFSM*. *Cladosporium* spp. mycelial growth was monitored in order to evaluate the morphological characteristics of the pathogen. The use of *Trichoderma* spp. as biocontrol was assessed through dual culture tests and production of volatile metabolites. *Trichoderma* spp. showed positive results in inhibiting the growth of *Cladosporium* spp., thereby presenting potential to be used as a biocontrol agent.

Keywords: biological control, dual culture tests, antibiosis, biocontrol agents.

1. INTRODUCTION

Caryaillinoinensis (Wangenh) C. Koch is commonly known as a pecan tree, and is a fruit species of temperate climate native to North America, where it is naturally established close to water courses. In Brazil, the species cultivation took place between the 1960s and 1970s, when orchards were implanted through fiscal incentives, mainly in the Southern and Southeastern regions (Ortiz & Camargo, 2005). Its cultivation has currently been expanding, and therefore information regarding diseases that affect the species is necessary.

One of the diseases recently reported in pecan trees is the leaf spot caused by species of the genus *Cladosporium*, including *C. pseudocladosporioides*, *C. cladosporioides*, and *C. subuliforme*. The main symptoms found are dark circular spots (Walker et al., 2016a), which affect leaves and fruits. Another species of this genus, *Cladosporium caryigenum*, has been associated with pecan scab, which is the main disease that attacks this crop (Ortiz & Camargo, 2005).

Considering that the pecan leaf spot (*Cladosporium* spp.) has affected cultivars and that there are no commercial products for disease control registered for this crop, there is lack of research in this area. Biological control appears as an interesting possibility, considering that it is a control method less harmful to the environment.

Among the most used antagonistic fungi to control pathogens, species of the genus *Trichoderma* stand out, which are natural soil inhabitants and present characteristics like fast growth and abundant sporulation (Howell, 2003). They act as biocontrol agents through some mechanisms such as antibiosis, which is characterized by the production of volatile or non-volatile antibiotic substances with fungicidal effects capable of inhibiting the mycelium growth; mycopredatism, which involves the action of enzymes capable of causing the death of one of the fungi that will serve as food for the survivor; and the competition for space and nutrients, represented by the dispute in search of food, physical space, and sunlight (Benítez et al., 2004; Ribeiro, 2009; Dias et al., 2013).

Thus, the present study aimed to evaluate the antagonist potential by *in vitro* tests through dual culture test and the production of volatile metabolites by *Trichoderma* spp. on *Cladosporium* spp., which is the causal agent of the leaf spot disease in *Caryaillinoinensis*.

2. MATERIAL AND METHODS

Three *Cladosporium* spp. isolates (6SC, 12/7PR and 16/2RS) provided by the "ElocyMinussi" Laboratory of Phytopathology - Federal University of Santa Maria were used in this study, which were stored according to the method of Castellani (distilled water) (Castellani, 1939) and previously purified in monosporic culture.

Isolates used were pathogenic to pecan trees, which were obtained from leaves that presented leaf spot symptoms collected from orchards located in municipalities of the three states of southern Brazil (Table 1) (Walker et al., 2016b).

Two commercial products were used for tests using biocontroller: Biotrich® (from *Trichoderma* spp.), Ecotrich® (from *T. harzianum*) and one isolate (T4 UFSM), made available by the fungi collection of the "Elocy Minussi" Laboratory, Federal University of Santa Maria, which was obtained from an analysis of the fungal population of soil classified as a Sand Loam Soil (Durigon et al., 2014).

Table 1. *Cladosporium* spp. isolates obtained from pecan tree leaf spots (*Caryaillinoinensis*) in southern Brazil.

Isolate	Species	Collection Date	Municipality/State	Coordinates (GMS)	GenBank Access Number
6SC	*Cladosporium pseudocladosporioides*	Jan/14	Palmitos – SC	-27°12'00" S, -53°22'10" W	KT991538
12/7PR	*Cladosporium cladosporioides*	Mar/14	Porto Amazonas – PR	-25°32'22" S, -49°54'24" W	KT991547
16/2RS	*Cladosporium subuliforme*	Apr/14	Cachoeira do Sul – RS	-30°00'20" S, -53°52'00" W	KT995114

(Walker et al. 2016b).

2.1. Mycelial growth of Cladosporium spp.

It is possible to observe the time required for the growth of *Cladosporium* spp. isolates through the mycelial growth test. Thus, it was possible to estimate the incubation period for subsequent tests.

To do so, an aliquot of 0.10 µL of spore suspension of each *Cladosporium* spp. isolate, adjusted to 1 x 10^6 conidia/mL^{-1} was inoculated at the center of Petri dishes (80 mm diameter) containing Potato Dextrose Agar (PDA) culture medium. After inoculation, plates were transferred to *Biological Oxygen Demand* (B.O.D.) incubation chamber at temperature of 25ºC and 12-hour photoperiod. Four replicates were made, each consisting of one plate. After 24 hours of incubation, the diameter of colonies was measured, which were performed in diametrically opposite directions with the aid of a digital caliper, resulting in an average of two readings. Readings were performed daily and completed at 23 days when colony growth completely occupied the plaque diameter, and according to the fastest growing species.

Data were submitted to analysis of variance and analyzed by linear regression using the Sisvar version 5.3 statistical software (Ferreira, 2009).

2.2. Antagonism of Trichoderma spp. against Cladosporium spp. under direct confrontation

First, *Cladosporium* spp. isolates (6SC, 2/7PR and 16/RS) and the sources of *Trichoderma*spp. were grown in PDA medium for 7 days at 25°C and 12-hour photoperiod.

The spore suspension of *Cladosporium* spp. isolates was subsequently adjusted to 1 x 10^6 conidia/mL^{-1}. Subsequently, an aliquot of 0.10 µL of the spore suspension was inoculated into Petri dishes (80 mm in diameter) containing PDA, 0.5 cm away from the edges. Dishes were sealed and incubated at 25°C with 12-hour photoperiod for 7 days. After this period, 0.10 µL of the *Trichoderma* spp. spore suspension at the same concentration were added to the opposite side of the dish containing *Cladosporium* spp. isolates (adapted from Machado & Silva, 2013). Dishes were again sealed and transferred to incubation chamber at 25°C with 12-hour photoperiod for 7 days. Petri dishes inoculated with only the spore suspension of each pathogen isolate were used as control.

From the second day, the diameter of *Cladosporium* spp. isolates was daily measured throughout the incubation period in diametrically opposite directions with the aid of a digital caliper.

The following treatments were used: T1 - 6SC (*C. pseudocladosporioides*); T2 - 2/7PR (*C. cladosporioides*); T3 - 16/2RS (*C. subuliforme*); T4 - Biotrich® x 6SC; T5 -Biotrich® x 12/7PR; T6 - Biotrich® x 16/2RS; T7 -Ecotrich® x 6SC; T8 - Ecotrich® x 12/7PR; T9 - Ecotrich® x 16/2RS; T10 - T4UFSM x 6SC; T11 - T4UFSM x 12/7PR and T12 - T4UFSM x 16/2RS. Four replicates were used for each treatment, in which each replicate was composed of a Petri dish.

The mycelial growth inhibition percentage was calculated according to the formula (Equation 1):

$$\%\ of\ inhibition = \left[\frac{(R1 - R2)}{R1}\right] \times 100 \quad (1)$$

In which: R1 = radial growth of control; R2 = radial growth of treatment (Jeyaseelan et al., 2012).

The scale proposed by Bell et al. (1982) was also used, which assigns classes varying from 1 to 5 depending on the antagonist's performance. Class 1 is used when the antagonist occupies the entire Petri dish; class 2 when the antagonist covers 2/3 of the dish reaching part of the pathogen; class 3 when the antagonist and the pathogen overgrow up to the middle of the dish, and none of the fungus dominates the other; class 4, the pathogen grows up to approximately 2/3 of the dish, partially covering the antagonist; and class 5, the pathogen completely overgrows the dish.

The experimental design was completely randomized, and the means were compared by the Tukey test at 5% error probability, using the Sisvar version 5.3 statistical software (Ferreira, 2009).

2.3. Inhibitory effect of volatile metabolites produced by Trichoderma spp. on Cladosporium spp.

Assays were performed based on the methodology described by Dennis & Webster (1971) and adapted in order to assess the inhibitory potential of volatile metabolites produced by *Trichoderma*spp. The same treatments used for the dual culture test were performed.

First, the spore suspension of *Cladosporium* spp. isolates was adjusted to 1 x 10^6 conidia/mL^{-1}. Subsequently, a 0.10 µL aliquot of the spore suspension was inoculated

into Petri dishes (80 mm in diameter) containing PDA. Plates were then sealed and incubated for 7 days at 25°C with 12-hour photoperiod. After this period, 0.10 µL of the antagonist spore suspension were inoculated into the center of new Petri dishes with the same dimensions and also containing PDA medium. The lids of dishes containing *Cladosporium* spp. isolates were subsequently removed and the bases containing antagonists and pathogens were mixed together using Parafilm®. Bases were overlain so that the antagonist remained on the lower/bottom surface and dishes were then incubated under the same temperature and photoperiod conditions mentioned above. As control, base containing the pathogen overlapped with another containing only PDA medium was used.

Four replicates were used for each treatment, each replicate consisting of the union of two Petri dish bases. Daily pathogen colony measurements were carried out using a digital caliper throughout the 7 days of incubation. The experimental design was completely randomized and means were compared by the Tukey test at 5% error probability using the Sisvar version 5.3 statistical software (Ferreira, 2009).

3. RESULTS AND DISCUSSION

3.1. Mycelial growth of Cladosporium spp.

Analyzing the mycelial growth of different *Cladosporium* spp. isolates, it was verified that there is a growth disparity between 6SC and 16/2RS isolates, in which both obtained colony diameter of approximately 71 mm at 23 days of evaluation in relation to 12/7PR isolate, which at the same time period presented diameter of approximately 50 mm (Figure 1). Thus, it could be inferred that *C. cladosporioides* species (12/7PR) has lower growth rate than *C. pseudocladosporioides* (6SC) and *C. subuliforme* (16/2RS) species, which seems to be an important morphological characteristic that distinguish them.

According to Bensch et al. (2010), the three species used in this study are part of the *Cladosporium cladosporioides* complex, which includes similar species, but with some specific morphological characteristics capable of distinguishing them.

In analyzing the mycelial growth of *C. cladosporioides* species, Pereira et al. (2005) characterized isolates

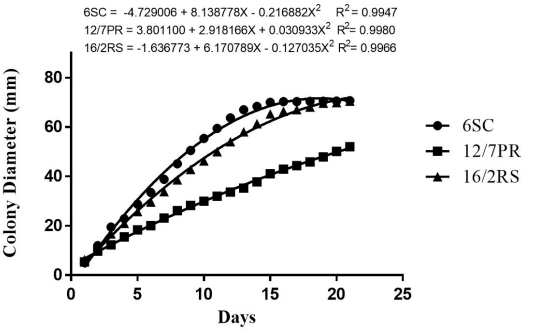

Figure 1. Regression analysis of *Cladosporium* spp. colonies cultivated in PDA culture medium at 25°C and 12-h photoperiod.

obtained from coffee tree fruits (*Coffea arabica* L.) and found that the diameter of colonies did not exceed 30 mm when grown in malt extract at 25°C for 10 days of incubation. Bataszkowski et al. (2005) cultivated *C. cladosporioides* isolates at room temperature and PDA culture medium and found colonies of approximately 55 mm for 10 days of incubation.

The test result using malt extract as culture medium and incubation temperature of 25°C for 10 days (Pereira et al., 2005) was similar to values obtained in the present study, where isolates cultured in PDA medium at 25°C presented mycelial growth of approximately 30 mm for 10 days of culture. However, in comparing the results obtained by Bataszkowski et al. (2005) using PDA culture medium and incubation at room temperature with results of this study, a difference can be verified, since the first study presented colonies of 55 mm of diameter for the same culture period, while colonies with diameter of 30 mm were observed in the present study. These differences in growth may occur due to factors such as composition of the culture medium, temperature and luminosity conditions, which are of paramount importance in determining the quality and quantity of characteristics such as mycelial growth and sporulation, and which are extremely important for the morphological differentiation of phytopathogens (Dhingra & Sinclair, 1995).

Bensch et al. (2010) analyzed the mycelial growth of *C. pseudocladosporioides* isolates grown in MEA (malt extract) and PDA over a 14-day period, which resulted in colony diameter ranging from 52 to 75 mm and from 65 to 78 mm, respectively. These results corroborated those obtained in the present study, in

which mycelial growth of about 65 mm was found for 14 days of incubation in PDA medium.

On the other hand and regarding *C. Subuliforme* species, no studies analyzing characteristics such as mycelial growth and sporulation were found in literature due to the recent discovery of the species (Walker et al., 2016a).

3.2. Dual culture tests between Cladosporium spp. and Trichoderma spp.

In dual culture tests, *Trichoderma* spp. isolate (T4UFSM) and Biotrich® and Ecotrich® compounds are potential biocontrol agents when evaluated by the class scale proposed by Bell et al. (1982). All treatments obtained class equal to 1.0, corresponding to the complete occupation of the dish by the antagonist (Table 2).

While testing the antagonistic action of bioprotectors based on *Trichoderma* spp. for controlling *Cylindrocladium candelabrum*, Maciel et al. (2012) concluded that *Trichoderma*spp.-based bioprotectors reached scores ranging from 1 to 2.25 according to the scale proposed by Bell et al. (1982), which proved to be efficient in the control of *Cladosporium* spp. isolated from pecan trees.

Ethur (2006) tested the antagonist potential of *Trichoderma* spp. isolates over *Fusarium solani* and *Fusarium oxysporum* isolates. The results found were considered efficient in reaching scores from 1 to 2.5 according to the scale of Bell et al. (1982). The author also classifies scores with values between 1 and 2 as very efficient for the *in vitro* control of pathogens.

By calculating the mycelial growth inhibition percentage of the antagonist over the pathogen (Table 3), it was observed that the lowest percentage value was found for 6SC isolate (*C. pseudocladosporioides*) with mean value of 16.48%, followed by 12/7PR (*C. cladosporioides*) and 16/2RS (*C. subuliforme*) isolates, with mean values of 18.38 and 28.14%, respectively. On the other hand, the compound from *Trichoderma* spp. with the greatest effectiveness in the test was Ecotrich®, inhibiting 20.87, 22.86 and 28.95% the mycelial growth of 6SC, 12/7PR and 16/2RS isolates, respectively.

Some authors report the effectiveness of using *Trichoderma* spp. as antagonist for inhibiting the mycelial growth of pathogens. Studies carried out by Silva et al. (2008), for example, consisted in evaluating the inhibition percentage of *T. stromaticum*, *T. viride*, *T. virens* and *T. harzianum*a gainst *Phytophthora citrophthora*, obtaining percentages of 52.21, 42.81, 30.35 and 15.18%, respectively. In testing the inhibition percentage at 8, 16 and 24 days of treatment of *Trichoderma* spp. isolates on *Mycosphaerellafijiensis* Morelet isolate, which causes Black Sigatoka in Banana (*Musa* spp.), Vega et al. (2006) found significant results ranging from 14.41 to 73.48%, 23.21 to 62.05% and 12.25 to 55.44% at 8, 16 and 24 days of evaluation, respectively.

The results previously mentioned corroborate those found in the present study, in which the Ecotrich® was able to inhibit 28.95% of the pathogen growth corresponding to approximately 1/3, thus demonstrating the efficiency of *Trichoderma* as a biocontrol agent.

In relation to the mycelial growth of the pathogen in the direct confrontation test, significant difference in the mycelial growth of *Cladosporium* spp. in the presence of the antagonist was observed when compared to control (Figure 2).

Table 2. Averages of the dual culture test between *Trichoderma* spp. and *Cladosporium* spp. using the scale of scores proposed by Bell et al. (1982).

Cladosporium spp.	*Trichoderma*spp.		
	Biotrich®	Ecotrich®	T4UFSM
6SC	1.0	1.0	1.0
12/7PR	1.0	1.0	1.0
16/2RS	1.0	1.0	1.0

Table 3. Inhibition percentage of *in vitro* mycelial growth of *Cladosporium* spp. in the dual culture test with *Trichoderma* spp.

*Cladosporium*spp.	*Trichoderma* spp.			
	Biotrich®	Ecotrich®	T4UFSM	Test.
6SC	15.44	20.87	13.12	0
12/7PR	17.15	22.86	15.13	0
16/2RS	28.78	28.95	26.69	0

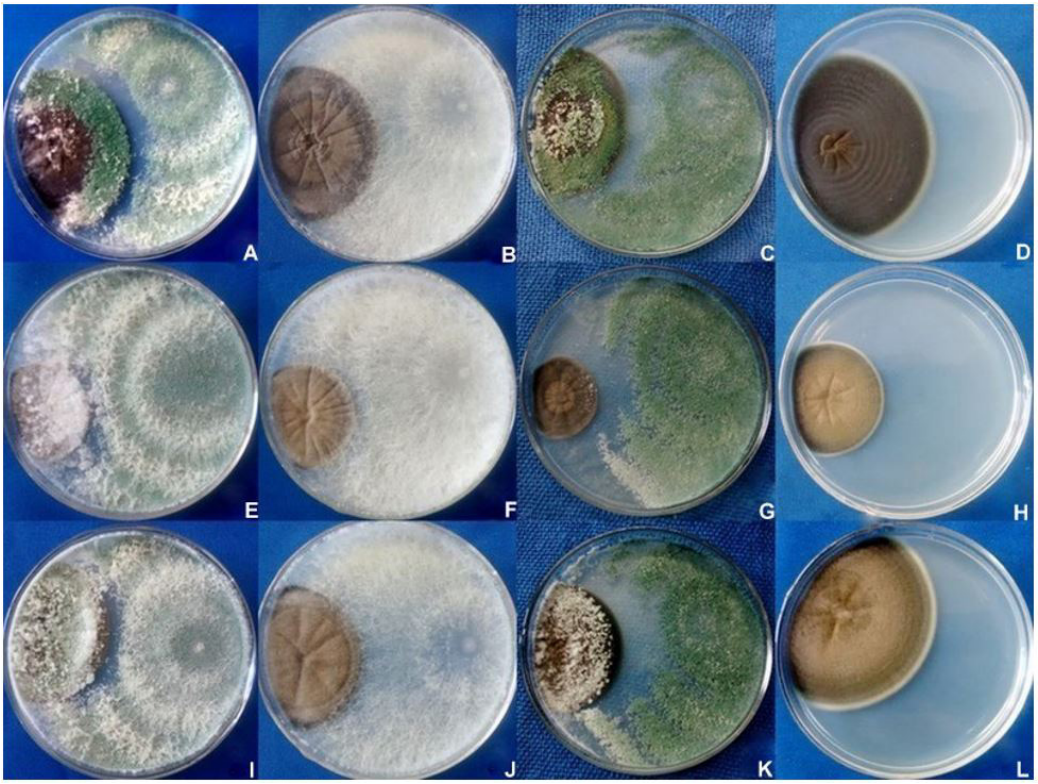

Figure 2. Dual culture tests using formulations and *Trichoderma* spp. isolate on the different *Cladosporium* spp. species in PDA medium. Where: Letters correspond to the different treatments used. "A": 6SC x Biotrich®; "B": 6SC x T4UFSM; "C": 6SC x Ecotrich®; "D": 6SC; "E": 12 / 7PR x Biotrich®; "F": 12 / 7PR x T4UFSM; "G": 12 / 7PR x Ecotrich®; "H": 12 / 7PR; "I": 16 / 2RS x Biotrich®; "J": 16 / 2RS x T4UFSM; "K": 16 / 2RS x Ecotrich®; "L": 16 / 2RS.

The 6SC isolate (*C. pseudocladosporioides*) presented greater mycelial growth, statistically differing from the other species, with values from 43.34 to 47.58 mm when compared to Ecotrich® and the T4UFSM isolate, respectively, in comparison to control, with 54.36 mm. However, *Trichoderma* spp. isolates did not differ statistically among themselves with the exception of when tested with 6SC isolate, where commercial product Ecotrich® was superior to T4UFSM isolate; however, they did not differ statistically from Biotrich® and all were considered effective when compared to control (Table 4).

Silva et al. (2014) tested the *in vitro* antagonist potential of *Trichodermalongibrachiatum*, *T. harzianum* and *T. viride* on *Fusarium solani* isolates and obtained expressive results. All *Trichoderma* species presented significant antagonistic effect against *F. solani* isolates, in which control obtained an exceptionally larger mycelial growth value when compared to those obtained by cultures in the presence of the antagonist agent. This result corroborates those obtained in the studies performed by Bomfim et al. (2010), who tested the *in vitro* antagonism of *T. viride*, *T. harzianum*, *T. stromaticum* and *T. virens* against *Rhizopus stolonifer* isolates, where *T. viride* had greater inhibition of the pathogen growth, while *T. stromaticum* and *T. virens* had lower inhibition potential when compared to the other species. Nevertheless, all biocontrol agents differed from control. These reports demonstrated the efficiency of *Trichoderma* spp. as a biological control for several phytopathogens.

3.3. The action of Trichoderma spp. volatile metabolites on Cladosporium spp. species

Regarding the production of volatile metabolites, all compounds based on *Trichoderma* spp. were efficient, at least for some of the different *Cladosporium* spp. species tested (Figure 3).

Table 4. *In vitro* mycelial growth of *Cladosporium* spp. in the dual culture test with *Trichoderma* spp. isolates.

Cladosporium spp.	*Trichoderma* spp.			
	Biotrich®	Ecotrich®	TUFSM	Test.
6SC	45.63 aBC*	43.34 aC	47.58 aB	54.36 aA
12/7PR	29.55 cB	27.52 cB	30.27 cB	35.67 bA
16/2RS	38.71 bB	38.62 bB	39.85 bB	54.36 aA
C.V	4.23			

(*) means followed by the same lower-case letter in vertical lines and upper-case letters in horizontal lines do not differ from each other by the Tukey test at 5% error probability; C.V.: variation coefficient.

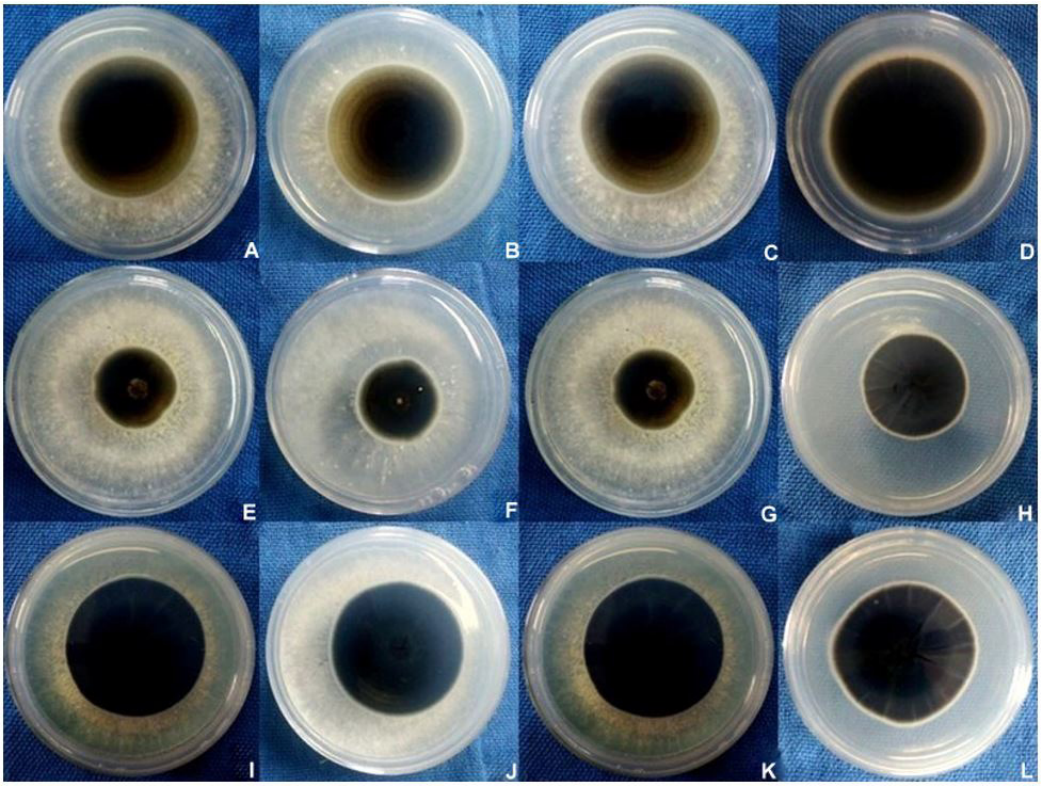

Figure 3. Volatile metabolites test using formulations isolated from *Trichoderma* spp. on different *Cladosporium* spp. isolates Where: Letters correspond to the different treatments used. "A": 6SC x Biotrich®; "B": 6SC x T4UFSM; "C": 6SC x Ecotrich®; "D": 6SC; "E": 12 / 7PR x Biotrich®; "F": 12 / 7PR x T4UFSM; "G": 12 / 7PR x Ecotrich®; "H": 12 / 7PR; "I": 16 / 2RS x Biotrich®; "J": 16 / 2RS x T4UFSM; "K": 16 / 2RS x Ecotrich®; "L": 16 / 2RS.

Commercial product Biotrich® and T4UFSM isolate showed the best results for the inhibition of the mycelial growth of *C. pseudocladosporioides* species (6SC); in addition, Ecotrich® also differed statistically from control. All compounds for the *C. cladosporioides* species (12/7PR) showed to be efficient; moreover, the species showed smaller mycelial growth when compared to the other species, while only T4UFSM isolate inhibited the mycelial growth of *C. subuliforme* (16/2RS), since the other products did not significantly differ from control, showing to be ineffective in this case (Table 5).

Using similar methodology, Gomes et al. (2001) obtained reduction in the mycelial growth of *Cylindrocladiumspathulatum*, which is responsible for the leaf spot in yerba mate (*Ilex paraguariensis* St. Hil), through volatile metabolites released by *Trichoderma* spp. The compounds released by the antagonist were also efficient when tested on *Sclerotiniasclerotiorum* isolates, considerably reducing its mycelial growth

Table 5. *In vitro* mycelial growth (mm) of *Cladosporium* spp. in the volatile metabolites test produced by *Trichoderma* spp.

Cladosporium spp.	*Trichoderma* spp.			
	Biotrich®	Ecotrich®	T4UFSM	Test.
6SC	41.89 aC*	47.87 aB	41.46 aC	59.95 aA
12/7PR	28.56 bB	28.69 bB	28.63 bB	35.42 cA
16/2RS	44.63 aBC	49.28 aA	40.61 aC	48.83 bAB
C.V.	5.62			

(*) means followed by the same lower-case letter in vertical lines and upper-case letters in horizontal lines do not differ from each other by the Tukey test at 5% error probability; C.V.: variation coefficient.

(Dildey et al., 2014). In addition, the results obtained in the present study corroborate those found by Dennis & Webster (1971), who reported that *Trichoderma* species is effective in the production of volatile gases capable of inhibiting the growth of several microorganisms in culture medium.

4. CONCLUSIONS

Trichoderma spp. is efficient in the *in vitro* control of *Cladosporium pseudocladosporioides, C. cladosporioides* and *C. subuliforme* species. In addition, there is reduction in the mycelial growth of pathogens through the production of volatile compounds produced by *Trichoderma* spp. biocontrol agent. Thus, *Trichoderma* spp. presents potential to be used in the control of pathogens causing leaf spots in pecan trees (*Caryaillinoinensis*).

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