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ACTION OF *Trichoderma asperellum* AND BIOACTIVE METABOLITES ON *Fusarium* spp., PATHOGENS OF *Carya illinoinensis*

Jéssica Emília Rabuske[1], Thiarles Brun[1], Mateus Alves Saldanha[1*], Laís da Silva Martello[2], Marcio Antonio Mazutti[3], Marlove Fátima Brião Muniz[2]

[1]Universidade Regional Integrada do Alto Uruguai e das Missões, Campus Frederico Westphalen, Frederico Westphalen, RS state, Brasil. jessicaemilia@uri.edu.br, brun@uri.edu.br.
[2]Universidade Federal de Santa Maria, Departamento de Defesa Fitossanitária, Santa Maria, RS state, Brasil. mtsmateusalves@gmail.com, laismartello@hotmail.com, marlovemuniz@yahoo.com.br.
[3]Universidade Federal de Santa Maria, Departamento de Engenharia Química, Santa Maria, RS state, Brasil. marciomazutti@gmail.com.

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Resumo

Ação de Trichoderma asperellum e de metabólitos bioativos em Fusarium spp., patógenos de Carya illinoinensis. O cultivo de nogueira-pecã [*Carya illinoinensis* (Wangenh.) K. Koch.] está em expansão no estado do Rio Grande do Sul e com isso houve aumento na ocorrência de doenças. Entre as doenças identificadas, está a podridão de raízes causada por *Fusarium* spp. O controle de fungos fitopatogênicos presentes no solo é difícil de ser obtido, e assim, a utilização de microrganismos antagonistas, como *Trichoderma* spp., se apresenta como uma forma viável de manejo. Assim, o objetivo do trabalho foi analisar as diferentes formas de ação de *Trichoderma asperellum* no controle de *Fusarium* spp. A ação antagônica foi avaliada por pareamento de culturas, presença de metabólitos voláteis e não voláteis. A inibição do crescimento micelial no pareamento de culturas variou de 10,38% a 43,17%, enquanto que para os metabólitos voláteis a inibição foi menor, alcançando valores de no máximo 14,54%. Quanto aos metabólitos não voláteis, a percentagem de inibição variou de zero para alguns tratamentos até 44,99% no controle de *Fusarium graminearum*. *T. asperellum* inibiu o crescimento de *Fusarium* spp. através dos diferentes modos de ação estudados.
Palavras-chave: podridão de raiz, confronto direto, metabólitos voláteis, metabólitos não voláteis.

Abstract

The cultivation of pecan tree [*Carya illinoinensis* (Wangenh.) K. Koch.] is expanding in Rio Grande do Sul state and, with that, there has been an increase in the disease reports. Among the diseases identified, there is a root rot caused by *Fusarium* spp. The control of phytopathogenic fungi present in the soil is difficult to obtain, and the control through antagonists such as *Trichoderma* spp. is a viable way of management. Thus, the objective of this work was to analyze different forms of action of *Trichoderma asperellum* in the control of *Fusarium* spp. The antagonistic action was evaluated by paring cultures, presence of volatile and non-volatile metabolites. The mycelial growth inhibition in the culture pairing ranged from 10.38% to 43.17%, while for volatile metabolites protection was lower, reaching values of a maximum of 14.54%. As for the non-volatile metabolites, a percentage of inhibition varied from zero for some treatments to 44.99% in the control of *Fusarium graminearum*. *T. asperellum* inhibited the growth of *Fusarium* spp. through different studied modes of action.
Keywords: root rot, direct confrontation, volatile metabolites, non-volatile metabolites.

INTRODUCTION

The pecan tree [*Carya illinoinensis* (Wangenheim) K. Koch] belongs to the Junglandaceae family, being a dual-purpose species and can be used in mixed production systems, intercropped with agricultural crops or in silvopastoral systems (WELLS, 2018). In the last two decades, the area dedicated to pecaniculture in the state of Rio Grande do Sul has increased significantly due to the increasing consumption of nuts and a financial return considered satisfactory for producers. Brazil is the fourth largest producer of pecans in the world, totalling 1,700 tons of shelled nuts (INC, 2021). However, with the growth of the planted area, an increase in the number of diseases reported for the crop was also observed.

In this sense, the genus *Fusarium* was described as a pathogen associated with losses in the nurseries which produce pecan seedlings. The pathogen attacks plants, causing root rot followed by nutritional deficiency, aerial part wilting and leaf necrosis, symptoms that reflect the disease. In sowing, losses of up to 10% of seedlings are reported and when the pathogen damage extends to the field, the attacked plants have reduced initial development (LAZAROTTO *et al.*, 2014).

Once they are aggressive, non-specific and cosmopolitan fungi, the control of *Fusarium* spp. is difficult to obtain (MACIEL *et al.*, 2017) and the use of chemical fungicides can cause environmental contamination, imbalance of natural soil biota, resistant strains of pathogenic microorganisms in addition to a decrease in the population of microorganisms responsible for nutrient cycling and antagonists (BOUBAKRI *et al.*, 2015).

Fungi of the genus *Trichoderma* are among the most studied microorganisms for biological control. Antagonists of the genus efficiently control a wide range of phytopathogens, including *Fusarium* spp. The control can be explained by several mechanisms of action such as ability to directly inhibit fungal structures, colonization of root surfaces and rhizospheric soil, increased plant growth, increased nutrient availability and induction of plant resistance to diseases (ZANDYAVARI *et al.*, 2024; YAO *et al.*, 2024).

Despite being efficient, the biological control faces several obstacles to its use, including the difficulty in registering products that follow the same registration procedures as a chemical product in addition to the shorter storage period, specific storage conditions, difficulties with formulation stability, possible incompatibility between ingredients, creating difficulties in *in vivo* application (MACHADO *et al.*, 2012).

Thus, according to Vinale *et al* (2014), the use of techniques that would make the formulation process more efficient, without necessarily keeping the microorganism alive, would optimize the potential of the bioproducts generated, both in controlling phytopathogens and in promoting the growth of plants. These bioproducts can be produced at low cost and in large quantities, making them easier to apply *in vivo*.

One way to obtain these bioproducts is by submerged fermentation. In this case, the microorganism is cultivated in a liquid medium, in which, in addition to the growth of its vegetative and reproductive structures, substances and enzymes are released that can be used for the most diverse purposes, including the induction of resistance, control of phytopathogens and promotion of the plant growth. Therefore, the objective of this work was to evaluate the different forms of action of *Trichoderma asperellum* antagonism in the development of *Fusarium* spp. by paired culture technique, bioactive and non-volatile metabolites.

MATERIAL AND METHODS

Origin and characterization of fungal isolates

For the experiments, three isolates of *Trichoderma asperellum* and three of *Fusarium* spp. were used. One of the *T. asperellum* isolates was obtained from a suspension of a commercial product with 2 x 109 conidia per mL plated onto the PDA medium (coded as Qt) and two from rhizospheric soil from an orchard of adult *Carya illinoinensis* plants, obtained through serial dilution up to 10^{-4} (coded as TR1 and TR4).

Isolates of *Fusarium* spp. were stored in the library of the Phytopathology Laboratory of the Federal University of Santa Maria, using *F. oxysporum*, *F. equiseti* and *F. graminearum*, coded as "UFSM – F17, UFSM – F18 and UFSM – F20", respectively. The morphological, molecular identification and pathogenicity of *Fusarium* spp. isolates were carried out by Lazarotto *et al.* (2014a and 2014b). Table 1 contains detailed information about the isolates used in the present work.

Table 1. Species of *Fusarium* and of *Trichoderma* used in the current study.
Tabela 1. Espécies de *Fusarium* e de *Trichoderma* empregados no presente estudo.

Species and coding of the isolate	Coordinates - collection location	Code GenBank (TEF - 1α)/Product
Trichoderma asperellum TR1	29º43'29"S – 53º43'0,51"W	MK982653
Trichoderma asperellum TR4	29º43'29"S – 53º43'0,51"W	MN082152
Trichoderma asperellum Qt	-	URM – 5911
Fusarium oxysporum	29°43'13.0"S - 53°43'1.90"W	KF022242
Fusarium equiseti	28°53'54.7"S - 52°01'59.9"W	KF601580
Fusarium graminearum	28°53'54.7"S - 52°01'59.9"W	KF022238

Crop pairing

For the culture pairing, all fungal isolates were previously grown in PDA (potato-dextrose-agar) medium (7 days at 25 ± 2 ºC and 12 h photoperiod). The technique consisted of a disc of culture medium, 6 mm in diameter, containing *Fusarium* spp. mycelium, transferred to Petri dishes, also containing PDA medium, approximately 5 mm from the edge of the plate. Then, a 6 mm diameter disk of culture medium, containing mycelium from *T. asperellum* isolates, was transferred to the opposite position of each plate, with the control treatment consisting only of the pathogen. After setting up the experiment, the plates were incubated at 25 ± 2 °C with a 12-h photoperiod in BOD.

The experimental design used was completely randomized, in a 4x3 bifactorial analysis (antagonist: 3 isolates of *T. asperellum* and absence of *T. asperellum* x pathogen: 3 isolates of *Fusarium* spp.) with five replications, each replication being a Petri dish. Culture pairing assessment was performed on the sixth day, when the fastest growing isolate covered the entire surface of the medium on the plate.

The evaluation was carried out by measuring the diameter of the colonies in two directions, with the aid of a digital caliper, comparing the mycelial growth of each treatment with the control, calculating the percentage

of inhibition of the mycelial growth (PIC), as described by Balouiri *et al.*, (2016): I (%)=[(DC-DT)/DC]x100, where: I= percentage of inhibition; DC= control colony diameter (mm); DT= colony diameter of treatments (mm).

Action volatile metabolites

To evaluate the action of volatile metabolites from *T. asperellum* on *Fusarium* spp., two bases of Petri dishes containing BDA individually received discs (6 mm in diameter) of the pathogen and antagonist cultures, arranged in the center of the plates. Afterwards, the plates were joined laterally with transparent plastic film. As a control, two bases were superimposed, one containing the pathogen and the other just culture medium. The plates used in the experiment were incubated at 25 ± 2 °C with a 12-h photoperiod.

The design was completely randomized, in a 4x3 bifactorial analysis (Antagonist: 3 isolates of *T. asperellum* and absence of *T. asperellum* x Pathogen: 3 isolates of *Fusarium* spp.), with four replications. The evaluation of mycelial growth inhibition for *Fusarium* spp. it was on the fifth day, when the fastest growing mycelial isolate filled the surface of the medium in the Petri dish.

The evaluation was carried out by measuring the diameter of the colonies in two directions, comparing the mycelial growth of each treatment with the control, determining the percentage of inhibition of mycelial growth (PIC), with the formula: I(%)=[(DC-DT)/DC]x100, where: I= percentage of inhibition; DC= control colony diameter (mm); DT= colony diameter of treatments (mm) (BALOUIRI *et al.*, 2016).

Non-volatile metabolites

Optimization of fermentation of Trichoderma asperellum isolates

The fermentations of *T. asperellum* isolates were carried out in Erlenmeyer flasks with a capacity of 250 mL, with a volume of 100 mL of liquid culture medium used. The cultivation conditions were optimized using a Plackett-Burman experimental design, with 11 runs. The cultivation medium was composed without variation by 20 g/L of corn maceration water (AMM) and 7.5 g/L yeast extract, according to Junges *et al.*, (2018). The variables tested were pH (5, 6 and 7), sucrose concentration (50, 75 and 100 g/L), inoculum concentration (105, 106 and 107 conidia/ml) and rotation value (100, 140 and 180 rpm).

The culture medium had its pH adjusted with the aid of a digital pH meter and was then autoclaved at 120 ºC for 20 min. After cooling, 1 mL of the conidial suspension of *T. asperellum* isolates was inoculated into a laminar flow chamber. The spore concentration was obtained from the growth of *T. asperellum* isolates in Petri dishes (7 days, 25 ± 2 °C, 12 h photoperiod), to which water was added and the surface was scraped using a Drigalski loop, followed by adjustment of the suspension in a Neubauer chamber. Afterwards, they were placed in a Shaker-type orbital shaker under agitation previously defined by the treatments. The treatments are described individually in Table 2.

Table 2. Treatments of liquid culture media composed of sucrose, corn maceration water (AMM), yeast extract (EL), spore concentration, medium agitation and hydrogen potential (pH), for the growth of *Trichoderma asperellum* isolates.

Tabela 2. Tratamentos dos meios de cultura líquido compostos por sacarose, água de maceração de milho (AMM), extrato de levedura (EL), concentração de esporos, agitação do meio e o potencial de hidrogênio (pH), para o crescimento dos isolados de *Trichoderma asperellum.*

Treatment	pH	Sucrose (g/L)	Agitation (rpm)	Spores [ ]
1	7	50	100	10^7
2	7	50	180	10^5
3	7	100	180	10^5
4	5	100	180	10^7
5	7	100	100	10^7
6	5	50	180	10^7
7	5	100	100	10^5
8	5	50	100	10^5
9	6	75	140	10^6
10	6	75	140	10^6
11	6	75	140	10^6

After 96 h of incubation, the biomass was separated from the liquid part (to obtain the culture filtrates), carried out by filtration through a 12 micron millipore membrane and then a 0.22 micron membrane to ensure that the culture filtrate was free from fungal spores. In both cases, a vacuum pump was used to reduce the filtration time. The culture filtrates were frozen for later use in bioassays. The fermentations were carried out in triplicate, for each of the conditions.

Action of non-volatile metabolites

To test the action of the culture filtrates obtained in controlling pathogenic fungi, 8 mL of the filtrates were first added for every 80 mL of melting PDA medium, which was cooled to 45ºC in a laminar flow chamber. The culture medium added with the culture filtrates was transferred to Petri dishes, where a 6 mm diameter disc of the monosporic culture of pathogenic fungi was deposited in the center of each dish.

For the control, in place of the filtrate, water was added to the medium in the same proportion (10%). The sides of the plates were sealed with transparent plastic film (adapted from Isaias *et al.*, 2014). The plates used in the experiments were incubated in BOD at 25 ±2 °C with a 12 h photoperiod.

For the analyses, the experimental design was completely randomized, with treatments organized in a bifactorial 11x3 (The 11 runs of the Plackett-Burman test x T. asperellum isolates). The experimental design was the Plackett-Burman, with five replications per treatment, with each replication consisting of a Petri dish. The evaluation of culture pairing and the non-volatile metabolite action test was carried out on the sixth day after setting up the experiments when the fastest growing isolate covered the entire surface of the medium on the plate. The evaluation was carried out by measuring the diameter of the colonies in diametrically opposite directions, comparing the mycelial growth of the control with each treatment, calculating the percentage of inhibition of mycelial growth (PIC), according to the formula: I(%)=[(DC-DT)/DC]x100, where: I= percentage of inhibition; DC= control colony diameter (mm); DT= colony diameter of treatments (mm) (BALOUIRI *et al.*, 2016).

Statistical procedure

The data obtained in the previously mentioned tests were subjected to analysis of variance and the means were compared using the Skott-Knott test at a 5% probability of error, using the statistical program SISVAR 5.3 (FERREIRA, 2014).

RESULTS

Crop pairing

In direct comparison, the isolates of *Fusarium* spp. followed practically the same behavior for final colony diameter and percentage of inhibition of mycelial growth, as these are correlated variables. Regarding the mycelial growth, the isolate F20 (*F. graminearum*) was the one with the largest colony diameter on the fifth day, differentiating itself from the others, followed by F18 (*F. equiseti*) and F17 (*F. oxysporum*). For all the isolates, the control treatment had the largest final colony diameter, statistically differentiating itself from the others, where there was a pairing with *T. asperellum* isolates (Table 3).

Table 3. The mycelial growth and the percentage of inhibition of mycelial growth of *Fusarium oxysporum* (F17), *Fusarium equiseti* (F18) and *Fusarium graminearum* (F20) compared with isolates of *Trichoderma asperellum* (TR1, TR4 and Qt) in the culture pairing test.

Tabela 3. Crescimento micelial e percentual de inibição do crescimento micelial de *Fusarium oxysporum* (F17), *Fusarium equiseti* (F18) e *Fusarium graminearum* (F20) confrontados com isolados de *Trichoderma asperellum* (TR1, TR4 e Qt) no teste de pareamento de culturas

Treatment	Mycelial growth (mm)			Inhibition (%)		
	F17	F18	F20	F17	F18	F20
TR1	34,6 Bb	37,1 Bb	41,3 Ac	31,6 Ba	30,4 Ba	46,6 Aa
TR4	37,9 Bb	44,1 Ab	47,4 Ab	26,6 Ba	17,4 Bb	38,8 Ab
Qt	39,2 Ab	41,9 Ab	41,6 Ac	22,6 Ba	22,9 Bb	46,2 Aa
Witness	50,7 Ba	53,4 Ba	77,4 Aa	-	-	-
C.V. (%)	8,1			18,1		

* Averages followed by the same lowercase letter in the column and uppercase in the line do not differ significantly from each other, the 5% probability of error, by the Skott-Knott.C test. V. (%) = coefficient of variation.

Trichoderma asperellum reduced the mycelial growth of *Fusarium* spp. isolates, with interaction between the *Trichoderma* spp factors. X *Fusarium* spp., where the inhibition of mycelial growth ranged from 17.4% (F18xtr4) to 46.6% (F20XTR1). The best inhibition results were for the F20 isolated (*F. graminearum*) with

inhibition of 46.6%, 38.8%and 46.2%, for TR1, TR4 and QT (*T. asperellum*) respectively, these values being until the triple of what was found for the other isolates of *Fusarium* spp.

For the percentage of mycelial growth inhibition, F18 (*F. equiseti*) and F17 (*F. oxysporum*) demonstrated similar behavior, showing no statistical difference from each other.

Volatile metabolites

The isolated with lower mycelial growth in the evaluation of volatile metabolites (Table 4) was F17 (*F. oxysporum*) (51.26 mm), followed by F18 (*F. equiseti*) with intermediate growth of 60.73 mm and finally F20 (*F. graminearum*) It was the isolated who had the highest mycelial growth filling the entire surface of the culture medium in this period.

Table 4. Mycelial and percentage growth of *Fusarium oxysporum* (F17), *Fusarium equiseti* (F18) and *Fusarium graminearum* (F20) mycelial growth in the inhibition (TR1, TR4 and QT) in the volatile metabolite test.
Tabela 4. Crescimento micelial e percentual de inibição do crescimento micelial de *Fusarium oxysporum* (F17), *Fusarium equiseti* (F18) e *Fusarium graminearum* (F20) confrontados com isolados de *Trichoderma asperellum* (TR1, TR4 e Qt) no teste de metabólitos voláteis

Treatment	Mycelial growth (mm)			Inhibition (%)		
	F17	F18	F20	F17	F18	F20
TR1	53,5 Ca*	60,7 Bc	70,2 Ab	2,8 Bb	13,2 Aa	12,3 Aa
TR4	51,3 Ba	66,5 Ab	68,4 Ab	7,0 Ba	4,8 Bb	14,5 Aa
Qt	55,1 Ca	66,4 Bb	80,0 Aa	0,0 Ab	5,1 Ab	0,0 Ab
Witness	55,1 Ca	69,9 Ba	80,0 Aa	-	-	-
C.V. (%)		3,1			29,4	

* Averages followed by the same lowercase letter in the column and uppercase in the line do not differ significantly from each other, the 5% probability of error, by the Skott-Knott test. Where: C.V. (%) = coefficient of variation.

In the volatile metabolites test, some *T. asperellum* treatments inhibited the growth of *Fusarium* spp., with significant interaction between the factors. The highest reduction in mycelial growth of pathogen was observed in the treatment with isolated *F. graminearum* F20 confronted with the isolate of *T. asperellum* codified as TR4 (14.54%), and this treatment did not differ from TR1 isolate. For the isolated of *F. Equiseti* (F18), TR1 treatment presented higher reduction (13.2%) and for *F. Oxysporum*, TR4 (7.0%). Qt was the isolated of T. *asperellum* and did not affect the growth of *Fusarium oxysporum* and *Fusarium graminearum*.

Non -volatile metabolites

Table 5 presents the mycelial growth results of the eleven culture filters from the growth of isolated TR1 in submerged fermentation, tested against *Fusarium* spp. On the sixth day after the installation of the experiment, the isolated that showed the highest mycelial growth was the F20 (*Fusarium graminearum*), which filled the entire surface of the culture medium in this period (80 mm), followed by the isolated F18 (*Fusarium equiseti*) with 68 , 09 mm and by F17 (*Fusarium oxysporum*) with 63.72 mm growth.

Table 5. Effect of Isolated Culture Filters TR1 (*Trichoderma asperellum*) on mycelial growth and the percentage of inhibition of *Fusarium oxysporum* (F17), *Fusarium equiseti* (F18) and *Fusarium graminearum* (F20).
Tabela 5. Efeito dos filtrados de cultura do isolado TR1 (*Trichoderma asperellum*) no crescimento micelial e percentagem de inibição de *Fusarium oxysporum* (F17), *Fusarium equiseti* (F18) e *Fusarium graminearum* (F20)

Treatment	Mycelial growth (mm)			Inhibition (%)		
	F17	F18	F20	F17	F18	F20
1	49,86 Bc*	58,80 Bc	60,12 Ad	21,75 Aa	13,64 Bc	24,86 Ab
2	44,05 Bc	58,90 Ac	44,01 Be	30,89 Ba	13,49 Cc	44,99 Aa
3	60,63 Aa	53,91 Bd	57,85 Bd	4,84 Bc	20,83 Ab	27,69 Ab
4	56,38 Cb	66,29 Bb	80,00 Aa	11,52 Ab	2,64 Bd	0,00 Be
5	61,78 Ba	73,34 Aa	73,37 Ab	3,04 Ac	0,00 Ad	8,29 Ad
6	46,57 Bc	40,51 Ce	74,12 Ab	26,91 Ba	40,51 Aa	7,34 Cd
7	54,54 Bb	62,79 Bc	80,00 Aa	14,41 Ab	7,79 Ad	0,00 Be
8	46,37 Cc	60,69 Bc	66,85 Ac	27,23 Aa	10,87 Bc	16,43 Bc
9	45,11 Cc	65,65 Bb	77,02 Aa	29,21 Aa	3,58 Bd	3,7 Be

10	48,31 Cc	68,79 Ba	80,00 Aa	24,18 Aa	0,00 Bd	0,00 Be
11	47,26 Cc	66,71 Bb	78,69 Aa	25,83 Aa	0,00 Bd	1,63 Be
Witness	63,72Aa	68,09 Aa	80,00 Aa	-	-	-
C.V. (%)		5,50			16,47	

* Averages followed by the same lowercase letter in the column and uppercase in the line do not differ significantly from each other, the 5% probability of error, by the Skott-Knott test. Where: c.v. (%) = coefficient of variation.

The best results for the reduction of the mycelial growth caused by TR1 isolated to F17 (*F. oxysporum*) and F20 (*F. graminearum*) from T2 treatment, where the variables used were pH 7, agitation 180 rpm, sucrose 50 g/l and Inocle Concentration 105 Conidia/ML, inhibiting mycelial growth at 30.89% and 44.99%, respectively. For F17 (*F. oxysporum*), other treatments significantly compromised the mycelial growth, being T1, T6, T8, T9, T10 and T11 not statistically differing from T2, while for F20 (*F. graminearum*), the other treatments were statistically inferior.

For F18 (*F. equiseti*), the treatment that showed the highest percentage of mycelial growth inhibition was T6, where independent variables were pH 5, agitation 180 rpm, sucrose 50 g/l, inoculum concentration 107, with value of value 40.51% reduction. This result was higher than the others, which did not exceed 21% of mycelial growth reduction.

For the results of eleven culture filters from the growth of isolated TR4 in submerged fermentation, tested against *Fusarium* spp (Table 6), on the sixth day after the installation of the experiment, the isolate that presented the highest mycelial growth was F20 (*F. graminearum*), which filled the entire surface of the culture medium during this period (80 mm), following 65.78 mm and F17 (*F. oxysporum*) isolated 65.78 mm with 59.08 mm of growth.

Table 6. Effect of Isolated Culture Filters TR4 (*Trichoderma askallum*) on mycelial growth and percentage of inhibition of *Fusarium oxysporum* (F17), *Fusarium equiseti* (F18) and *Fusarium graminearum* (F20).
Tabela 6. Efeito dos filtrados de cultura do isolado TR4 (*Trichoderma asperellum*) no crescimento micelial e percentagem de inibição de *Fusarium oxysporum* (F17), *Fusarium equiseti* (F18) e *Fusarium graminearum* (F20)

Treatment	Mycelial growth (mm)			Inhibition (%)		
	F17	F18	F20	F17	F18	F20
1	45,52 Cb*	58,18 Bc	79,24 Aa	22,95 Ab	11,55 Bb	0,95 Cb
2	40,04 Cb	59,13 Bb	80,00 Aa	32,23 Aa	10,11 Bb	0,0 Bb
3	48,73 Bb	61,25 Ab	64,83 Ab	17,52 Ab	6,89 Bb	18,96Aa
4	42,44 Cb	61,00 Bb	80,00 Aa	28,16 Aa	7,27 Bb	0,00 Cb
5	55,68 Ca	56,25 Bc	80,00 Aa	5,75 Ac	14,49 Ab	0,00 Bb
6	58,25 Ba	70,15 Aa	75,00 Aa	10,41 Ac	0,00 Ac	6,25 Ab
7	46,74 Cb	62,97 Bb	80,00 Aa	20,88 Ab	4,27 Bc	0,00 Cb
8	43,93 Cb	51,18 Bc	77,66 Aa	25,64 Aa	22,19 Aa	2,92 Bb
9	54,32 Ba	56,89 Bc	76,04 Aa	8,06 Ac	13,52 Ab	4,95 Bb
10	56,94 Ba	55,16 Bc	80,00 Aa	3,62 Ac	16,14 Ab	0,00 Bb
11	58,09 Ba	54,69Bc	80,00 Aa	1,68 Ac	16,86 Ab	0,00 Bb
Witness	59,08 Ba	65,78 Ba	80,00 Aa	-	-	-
C.V. (%)		6,85			27,2	

* Averages followed by the same lowercase letter in the column and uppercase in the line do not differ significantly from each other, the 5% probability of error, by the Skott-Knott test. Where: C.V. (%) = coefficient of variation.

In the percentage of the mycelial growth reduction, it was observed that there was a statistical difference between treatments and between isolates. The treatments that demonstrated the best results regarding the inhibitory effect on mycelial growth of F17 (*F. oxysporum*) were T2, T4 and T8 with values of 32.23, 28.16, 25.64%, while for F18 (*F. equiseti*), the best treatment was T8 (22.19%).

For F20 (*F. graminearum*), in general, the percentage of mycelial growth reduction was lower than for the other isolates, even revealing treatments without effect, such as T4, T5, T7, T10 and T11. Treatment with the highest inhibition was T3 (18.96%), which statistically differentiated from the others to the isolated.

According to Table 7, on the sixth day after the installation of the experiment, the isolated that presented lower mycelial growth was the F17 (*F. oxysporum*) (61.58 mm), followed by F18 (*F. equiseti*) with intermediate

growth of 68.34 mm and finally F20 (*F. graminearum*) being the isolated that presented the highest mycelial growth filling the entire surface of the culture medium in this period.

Table 7. Effect of QT (*Trichoderma asperellum*) culture filters on mycelial growth and percentage of inhibition of *Fusarium oxysporum* (F17), *Fusarium equiseti* (F18) and *Fusarium graminearum* (F20).
Tabela 7. Efeito dos filtrados de cultura do isolado Qt (*Trichoderma asperellum*) no crescimento micelial e percentagem de inibição de *Fusarium oxysporum* (F17), *Fusarium equiseti* (F18) e *Fusarium graminearum* (F20).

Treatment	Mycelial growth			Percentage of inhibition		
	F17	F18	F20	F17	F18	F20
1	49,76 Aa*	69,48 Ba	80,00 Aa	19,19 Ab	0,00 Bc	0,00 Bb
2	60,62 Aa	69,79 Aa	77,61 Aa	1,56 Ac	0,00 Ac	2,98 Ab
3	58,21 Ba	60,94 Ba	78,76 Aa	5,47 Ac	10,83 Ab	1,55 Bb
4	53,15 Ba	58,71 Ba	80,00 Aa	13,68 Ab	14,09 Ab	0,00 Bb
5	51,89 Aa	67,33 Aa	63,26 Ab	15,74 Ab	1,48 Bc	20,92 Aa
6	65,74 Aa	64,33 Aa	69,64 Aa	0,00 Bd	5,87 Bb	12,95 Aa
7	62,82 Ba	61,39 Ba	78,02 Aa	0,00 Bd	10,17 Ab	2,47 Bb
8	40,09 Cb	62,37 Ba	80,00 Aa	34,9 Aa	8,7 Bb	0,0 Bb
9	51,25 Aa	51,66 Aa	65,10 Ab	16,77 Ab	24,41 Aa	18,62 Aa
10	58,55 Aa	54,98 Aa	61,57 Ab	4,9 Bc	19,55 Aa	23,04 Aa
11	53,61 Aa	56,39 Aa	59,74 Ab	12,94 Ab	17,48 Aa	25,32 Aa
Witness	61,58 Aa	68,34 Aa	80,00 Aa	-	-	-
C.V. (%)	13,19			28,23		

* Averages followed by the same lowercase letter in the column and uppercase in the line do not differ significantly from each other, the 5% probability of error, by the Skott-Knott test. Where: c.v. (%) = coefficient of variation.

For the percentage of inhibition of mycelial growth, it was observed that there was a statistical difference between treatments and between isolates. For F17 (*F. oxysporum*), the treatment that demonstrated the best effect was T8 (34.9), where the variables measured during growth were pH 5, agitation 100 rpm and sucrose 50 g/L.

The best treatment in inhibiting mycelial growth of isolate F17 (*F. oxysporum*) differed statistically from the other treatments. While for F18 (*F. equiseti*) and F20 (*F. graminearum*), the central point of the design (pH 6, Agitation 140 rpm, Sucrose 75 g/L, the inoculum concentration 106) did not differ from T8, with significant values in reduction of mycelial growth, and reduction of mycelial growth results 24.41 and 25.32%, respectively.

DISCUSSION

In the present study, different control mechanisms are analyzed with which three isolates of *T. asperellum* (TR1, TR4 and Qt) can act on fungi of the genus *Fusarium*, which cause root rot in pecan trees, and for this purpose, different tests. The first was the pairing of cultures and in this test, the inhibition of mycelial growth of *Fusarium* spp. ranged from 10.38% (F18xTR1) to 43.17% (F20xTR4), as in general, *T. asperellum* isolates show faster mycelial growth and are thus able to inhibit the growth of phytopathogenic fungi, as observed in the present research. This fact can be explained because the *Trichoderma* fungus uses different mechanisms for its survival and proliferation, including competition, which includes the rapid degradation and use of complex carbohydrates, thus quickly colonizing the habitats where it is found, preventing the colonization and survival of the phytopathogens fungi.

The reduction in mycelial growth of *Fusarium* spp. by *Trichoderma* spp. in the culture pairing test was reported by some authors, such as Das, Haridas and Sabu (2019), who, working with *T. harzianum* to control *F. oxysporum*, which causes soft rot in ginger (*Zingiber officinale* Rosc.), found values of up to 78.3% inhibition of mycelial growth, values higher than those found in present research. On the other hand, Silva *et al.* (2019), studying the inhibition of mycelial growth of *Fusarium subglutinans* isolated from *Pinus* spp. seeds by *Trichoderma* spp., found lower mycelial growth inhibition values, ranging from 11.5 to 38.4%.

In the crop pairing test, in addition to competition, other control mechanisms are acting, including parasitism. In this control mechanism, *Trichoderma* spp. directly parasitizes the structures of the phytopathogen, through penetration and colonization of the hyphae. Also, the action of hydrolytic enzymes and secondary metabolites act to control the pathogen, thus, the synergistic action of hyphae coiling, the production of antibiotics and enzymes (antibiosis), at the same time explains the efficiency of controlling phytopathogens by fungi of the

genus *Trichoderma* (VINALE *et al.*, 2014). However, the culture pairing test cannot elucidate the importance of antibiosis in controlling *Fusarium* spp. *in vitro*. Therefore, it is necessary to carry out other tests, including volatile metabolites and non-volatile metabolites.

The inhibition by volatile metabolites was lower than the inhibition obtained in the culture pairing test, showing that *T. asperellum* isolates present variation in the control of phytopathogens, in the different mechanisms of action associated with each of the tests, as already observed in the work carried out by Isaias *et al.* (2014). The mechanism of action that can be attributed to the decrease in mycelial growth of pathogens, caused by volatile metabolites and culture filtrates, is antibiosis. Antibiosis is the result of the action of volatile and/or non-volatile metabolites produced by *Trichoderma* spp. in the pathogen control process, which inhibit or prevent its development.

Thus, culture filtrates obtained from *T. asperellum* isolates under different growth conditions inhibited the mycelial growth of different species of *Fusarium* spp. The condition 8 stands out (pH 5, Agitation 100 rpm, Sucrose 50 g/L, inoculum concentration 105 spores/mL), which adapted well to all *T. asperellum* isolates. In this growth condition, metabolites were generated that inhibited the mycelial growth of *Fusarium* spp. *in vitro*. The culture filtrate of *Trichoderma* spp. was described as efficient in reducing the mycelial growth of *Fusarium* spp. in other studies, such as that carried out by Cong *et al.* (2019), where the authors tested the fitrate of *Trichoderma* pseudokoningii culture cultivated in submerged fermentation, in the control of *F. oxysporum* f.sp. *cucumerinum*, which causes wilt in cucumber plants, and observed values of up to 51.52% in the reduction of mycelial growth of the phytopathogen.

Louzada *et al.* (2016), evaluating the action of *Trichoderma* spp. in the control of *Sclerotinia sclerotiorum* (Lib.) de Bary, by different methods, such as pairing cultures, volatile metabolites, non-thermo-labile metabolites (culture filtrates), observed that the best results of each test allowed only 16.5%, 9 .5% and 0.3% of the mycelial growth of the pathogen, respectively, demonstrating the efficiency of culture filtrates in controlling *S. sclerotiorum.* However, in the fermentation process, some parameters, such as time and environmental conditions, influence the production of secondary metabolites and according to Specian *et al.* (2014), optimizing antagonist cultivation conditions leads to the production of more effective compounds and should be part of the biological control program.

Among the variables used to optimize the cultivation of *T. asperellum* for the production of culture filtrates, the agitation stands out, and other authors, such as Soares and Maringoni (2018), comment on the importance of agitation of the cultivation medium in production of secondary compounds, These authors highlighted that agitation of the medium caused differentiation in the color of the product generated, probably due to the production of substances and pigments, and on the other hand, when the medium was not agitated, a white mycelial layer was produced on the surface of treatments. In addition to agitation, pH also interferes with the production of antifungal compounds, as observed by Cripps-Guazzone *et al.* (2016), where it was possible to observe that, regardless of pH, the production of metabolites with antifungal activity occurred, as occurred in the present study. According to Cripps-Guazzone *et al.* (2016), at different pH values, different compounds or enzymes are produced by *Trichoderma* spp. For example, for trypsin-type protease and a pH between 6.0-7.0 would be ideal. In the case of cellobiohydrolase and N-acetylglucosaminidase, the value would be 3.0, for ß-xylosidase 6.0 and for ß-glucosidase the optimal pH value for production would be 5.0.

According to Vinale *et al.* (2009), the use of metabolites from *Trichoderma* spp. obtained by mass cultivation can make it possible to obtain new biofertilizers and biopesticides based on these compounds, benefiting the management of plant diseases. In this sense, in *Trichoderma* spp. culture filtrates, there are many antimicrobial compounds that suppress or reduce the growth or proliferation of phytopathogenic fungi. Among these compounds are cell wall degrading enzymes, antibiotics and volatile compounds (MUHAMMAD ADNAN *et al.*, 2019). In the present study, the effectiveness of the action of these compounds in reducing the mycelial growth of *Fusarium* spp.

In the present work, it was also possible to verify that *T. asperellum* isolates use different mechanisms of action to control *Fusarium* spp. *in vitro*. Thus, Solino *et al.* (2017) stated that microorganisms that have the characteristic of presenting different modes of action, for example, in addition to parasitizing phytopathogen structures, they produce metabolites with a fungistatic or fungicidal effect, aspects that are added to biocontrol programs and can present ineffective biological control *in vivo* more efficiently.

CONCLUSION

● The fungus *Trichoderma asperellum* inhibited the growth of *Fusarium oxysporum*, *Fusarium equisetti* and *Fusarium graminearum* through direct confrontation and the production of volatile and non-volatile metabolites.

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