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ORIGINAL ARTICLE

Trichoderma asperellum in the biocontrol of *Lasiodiplodia theobromae* and *Pseudofusicoccum kimberleyense*

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Abstract

Lasiodiplodia theobromae and *Pseudofusicoccum kimberleyense* are pathogens causing trunk canker in *Carya illioniensis* and there are still no reports of effective forms of control. However, biological control is a promising measure. The objective of this work was to isolate, identify and evaluate the action of *Trichoderma* spp. in the *in vitro* control of *L. theobromae* and *P. kimberleyense* and to identify the compounds produced by the antagonist. *Trichoderma* spp. was identified by molecular technique and morphologically characterized. The antagonistic action of *T. asperellum* isolates (obtained from the rhizospheric soil, and of an isolate obtained from a commercial formulation) was evaluated by pairing cultures and volatile metabolites on *L. theobromae* and *P. kimberleyense*. Optimization of the cultivation method of *T. asperellum* was carried out and the compounds produced by the antagonist were identified by gas chromatography. Isolates obtained from the soil were identified as *T. asperellum* and decreased mycelial growth of *L. theobromae* and *P. kimberleyense* in the crop pairing test (48.98% S6 x Qt), as well as by volatile metabolites (29.85% SM21 x TR4). The cultivation conditions that generated the filtrates with the greatest antifungal action used 20 g · l^{-1} of corn maceration water, yeast extract 7.5 g · l^{-1}, pH 5, agitation 100 rpm, sucrose 50 g · l^{-1}, inoculum concentration 10^5 spores /ml. Among the identified compounds, some stood out for having bioactive action, such as pyran derivatives, celidoniol, deoxy, pentadecanoicacid, 2,3-dihydro-3,5-dihydroxy-6-methyl, propanoicacid, 1-methylethyl ester and 9-octadecenoic acid. The *T. asperellum* isolates showed potential for biocontrol *in vitro*, acting by different mechanisms.

Keywords: *Carya illinoinensis*, chromatographic analysis, culture filtrates; secondary metabolites

Introduction

Carya illinoinensis (Wangenh.) K. Koch, popularly known as pecan nut, is a species of the Junglandaceae family, native to North America and Mexico (Vázquez 2016). In Brazil, pecan is grown mainly in the south. This species has grown in economic importance because there has been an increase in area planted with pecan and, consequently, an increase in the number of diseases, mainly those caused by fungal pathogens.

These cause serious damage to the crop, such as reduced productivity, reduced quality of the final product and even the death of plants in the orchard. Among the diseases reported is stem canker caused by fungi of the Botryosphaeriaceae family. The symptoms of the disease are fissures or small elliptical lesions in the bark of the trunk and branches, which progress to cankers of up to 60 cm, with black streaks close to the vascular tissues and death of plants (Poletto *et al*. 2016; Rolim *et al*. 2020; Rolim *et al*. 2022).

Biological control with the genus fungi of *Trichoderma* is effective in controlling pathogens that can survive in the soil. These microorganisms have good adaptation to this environment, inhibiting the fungal structures of the pathogen, or colonizing root surfaces and rhizospheric soil. They also increase plant growth due to the availability of nutrients and make it less susceptible (Chen *et al*. 2019a; Sanchez *et al*. 2019).

In biological control, maintaining the antagonist alive is a condition to ensure control of the disease. The reproduction and optimization of compounds produced by these microorganisms in a culture medium, before the application of the bioproduct, makes it a viable alternative, with no need to keep the microorganism alive at the end of the process. Thus, only compounds released during fungal development are used for control, optimizing the potential of the generated bioproducts, both in the control of and in the promotion of plant growth. These bioproducts can be produced at low cost and in large quantities, making it easier to apply *in vivo* (Vinale *et al*. 2014).

One way of obtaining these bioproducts is through submerged fermentation. In this case, the microorganism is grown in a liquid medium, where in addition to the growth of its vegetative and reproductive structures, the fungus releases substances and enzymes that can be used for the most diverse purposes, including the control of phytopathogens and weeds (Chen *et al*. 2019b).

Therefore, the objective of this work was to obtain and identify isolates of *Trichoderma* spp. associated with *Carya illioniensis* rhizosphere, to evaluate the action of *Trichoderma* spp. in the *in vitro* control of *Lasiodiplodia theobromae* and *Pseudofusicoccum kimberleyense* and to identify bioactive compounds produced by *Trichoderma* spp.

Materials and Methods

Origin of isolates

Three isolates of *Trichoderma* spp. are antagonistic: two from rhizospheric soil in an orchard of *C. illinoinensis* plants (coded as TR1 and TR4), and an isolate obtained from an aliquot of commercial product with 2×10^9 conidia per ml, which was plated onto potato-dextrose-agar (PDA) medium (coded as Qt). As pathogens, two isolates that cause stem canker in pecan tree, *L. theobromae* and *P. kimberleyense*, both were from the library of the Phytopathology Laboratory of the Federal University of Santa Maria, and were coded as "S6" and "SM21" (Table 1).

Molecular characterization of *Trichoderma* spp.

The DNA of the isolates TR1 and TR4 was extracted by scraping the mycelial growth of the fungus, grown for 7 days in PDA culture medium, with the Extraction Kit ZR Fungal / Bacterial DNA Mini Prep (ZymoResearch, Irvine, CA, USA). Then part of the 1-alpha elongation factor (EF1) gene was amplified with the primer pair A-TEF_F (5 'CCTTCAAGTACGCYTGGGTTC-3') and A-TEF_R (5 '-TTCTTGGAGTCACCGGCAA-3') (Perrone *et al.* 2011).

The nucleotide sequences obtained were compared with those of the National Center for Biotechnological Information GenBank (http://www.ncbi.nlm.nih.gov/) (Altschul *et al*. 1997). The sequences of GenBank that presented the highest scores and of other species of *Trichoderma* were selected and aligned, with the sequences obtained in the sequencing of the isolates of the present research, by the ClustalW algorithm (Thompson *et al*. 1994). Phylogenetic analysis was

Table 1. Species and isolate coding, coordinates (GMS) of the place of collection, Genbank/product deposit code, SMDB access and Sisgen Code

Isolate species and coding	Coordinates (GMS) of the collection site	GenBank/ Product code	SMDB code	Sisgen code
Trichoderma asperellum (TR1)	29º43'29"S – 53º43'0,51"O	MK982653	18.371	A32646D
Trichoderma asperellum (TR4)	29º43'29"S – 53º43'0,51"O	MN082152	18.372	A32646D
Trichoderma asperellum (Qt)	–	URM – 5911	–	–
Lasiodiplodia theobromae (S6)	29º21'06"S – 52º58'39"O	MT533179	18.362	A32646D
Pseudofusicoccum kimberleyense (SM21)	29º41'05,12"S – 53º43'33,16"O	MH188485	18.364	A32646D

performed using the "neighbor–joining" statistical method with 1000 replicates, using the MEGA version 4 program (Tamura *et al*. 2007). The similarity of the nucleotide sequences between the isolates was calculated using the basic local alignment search tool – BLAST procedure. After identification, the sequences of the present research were deposited in GenBank.

Morphological characterization of *Trichoderma* spp.

The mycelial growth rate (MGR) was determined by transferring discs (6 mm, PDA medium) of isolates of *Trichoderma* spp., obtained from colonies with 7 days of growth, to other plates with PDA medium (25 ± 2°C, with 12 h of photoperiod). The mycelial growth of each isolate was incubated and evaluated daily for 5 days, when the first repetition colonized the entire surface of the culture medium, two diametrically opposite positions of each plate were measured. For the final average diameter of the colony, the diameter measured on the fifth day of mycelial growth evaluation was used.

Sporulation was evaluated after 7 days of incubation, by adding 10 ml of sterile distilled water, plus a drop of the emulsifier Tween 80®. The suspension was stirred for 30 s with the aid of a micropipette. One ml was removed and spread in a Neubauer chamber for later determination of the conidia concentration (conidia · ml^{-1}). Colony coloration was observed after 7 days of incubation, with the aid of the Munsell -Soil Color Chart (Munsell 2009) and the conidia were characterized by measuring the width and length of 30 conidia.

Direct confrontation and volatile metabolites

PDA culture medium disks containing mycelium from pathogenic isolates were transferred to Petri dishes containing PDA medium. Then, a PDA disk, containing mycelium from *Trichoderma* spp. isolates, was transferred to the opposite position of each plate. As a control, only the pathogen on the plate was used. All fungal isolates used in the experiment were previously cultured in PDA, for 7 days in BOD (25 ± 2°C, photoperiod 12 h). After the installation of the experiment, the plates were incubated (25 ± 2°C, photoperiod 12 h).

The inhibitory effect of volatile metabolites was assessed as described by Dennis and Webster (1971), with adaptations. In this methodology two bases of Petri dishes of 90 mm containing PDA received, individually, disks (6 mm) of the cultures of the pathogen and the antagonist, arranged in the center of them. Afterwards, these were superimposed and joined laterally, with transparent plastic film. As a control, two bases were superimposed, one containing the pathogen and the other only culture medium. The plates used in the experiment were incubated in BOD (25 ± 2°C, photoperiod 12 h).

The experimental design of the culture and volatile metabolites pairing tests was completely randomized, in a 4 × 2 bifactorial analysis (Antagonist: 3 isolates of *Trichoderma* spp. and absence of *Trichoderma* spp. x Pathogen: *L. theobromae* and *P. kimberleyense*). The evaluation of mycelial growth inhibition in the volatile metabolites experiment was carried out on the fourth day after the installation of the experiment and of pairing cultures on the fifth day. The evaluation of treatments was performed by measuring the diameter of colonies in two axes, comparing the radial growth of each treatment with the control, calculating the percentage of inhibition of mycelial growth (PIC), according to equation: I(%) = [(DC–DT)/DC] × 100, where: I = percentage of inhibition; DC = control colony diameter (mm); DT = colony diameter of treatments (mm) (Isaias *et al*. 2014).

Optimization of fermentation of isolates of *Trichoderma* spp.

The cultivation conditions were optimized by means of a Plackett-Burman experimental design, with 11 runs with three central points. The variables tested were pH (5, 6 and 7) sucrose (50, 75 and 100 g · l^{-1}), inoculum concentration (10^5, 10^6 and 10^7) and agitation (100, 140 and 180 rpm). The medium was supplemented with 20 g · l^{-1} of corn maceration water (AMM) and 7.5 g · l^{-1} yeast extract (Junges *et al*. 2018). When the pH was below the desired level, sodium hydroxide was used to increase the pH and when it was above, hydrochloric acid was used to lower it, thus adjusting the pH value.

The Erlenmeyers containing the culture medium were autoclaved (120°C for 20 min.). After cooling, the culture medium was inoculated with 1 ml of the spore suspension of *Trichoderma* spp. in a laminar flow chamber. The spore concentration was obtained from the growth of *Trichoderma* spp. in Petri dishes (7 days, 25 ± 2°C, 12 h photoperiod), in which distilled and sterilized water was added and the colony surface was scraped with a Drigalski loop, followed by adjustment of the suspension in a Neubauer chamber. Then, the treatments were placed in an incubator chamber with orbital agitation, under agitation speed previously defined by the treatments.

After 96 h of incubation, the biomass was separated from the liquid part, obtaining the culture filtrates, performed by filtration in a 12 µm millipore membrane and then in 0.22 µm, using a membrane filtration system coupled to a water pump vacuum to reduce filtration time. The culture filtrates were frozen for later use in the experiments.

Non-volatile metabolites of *Trichoderma asperellum* in the inhibition of mycelial growth of *Lasiodiplodia theobromae* and *Pseudofusicoccum kimberleyense*

The culture medium with the added filtrates (10 v/v im PDA medium flux) was homogenized and then poured into Petri dishes, where a 6 mm disk of the pathogenic fungi was placed in the center of each dish. For the control, sterile distilled water was added to the culture medium in the same proportion (10%), replacing the culture filtrate (Isaias *et al*. 2014). The sides of the plates were sealed with clear plastic film. The plates used in the experiments were incubated (25 ± 2°C with a 12 h photoperiod).

The experimental design was the Plackett-Burman. The evaluation of the action test of non-volatile metabolites was on the fifth day after installation of the experiments when a repetition occupied the entire surface of the medium. The evaluation was carried out by measuring the diameter of the colonies in two axes, comparing the mycelial growth of each treatment with the control. Finally, the percentage of inhibition of mycelial growth (PIC) was calculated (Isaias *et al*. 2014).

Chromatographic analysis of the culture filtrates of *Trichoderma* spp.

For liquid-liquid extraction, 10 ml of the culture filtrates of *Trichoderma* spp. grown under condition eight were mixed with 50 ml of the solvents: methanol, ethanol and hexane. The mixing was carried out in a volumetric flask and the resulting solutions were stirred for 24 h at room temperature. After this period, the solutions were placed in a test tube for 24 h (ethanol and methanol) and 1 h (hexane). The supernatant was stored in a refrigerator for analysis.

The molecules produced were determined using a multidimensional gas chromatography system (Shimadzu, model MDGC / GCMS-2010) equipped with a mass spectrometry detector (QP-2010 Ultra) and flame ionization detector (FID-2010 Plus), and automatic injection system (AOC-20i).

The samples used1 µL, which was injected in split mode (30: 1) at 250°C. Fused silica column Rtx®-5ms GC (30 m × 0.25 mm id × 0.25 µm film thickness) was used with 5% diphenyl and 95% dimethylpolysiloxane (Restek Corporation, Bellefonte, PA, USA). Helium gas was used for transport at a flow rate of 1.69 ml · min^{-1} for samples dissolved in hexane and 1.33 ml · min^{-1} for those dissolved in methanol and ethanol. The injection temperature was 270°C for all samples analyzed.

For samples dissolved in hexane, the oven temperature was increased from 50°C to 280°C at 5°C/min, remaining so for 15 min. In the case of samples in methanol and ethanol, the oven temperature was increased from 100°C to 310°C at 5°C · min^{-1}, remaining so for 15 min. The temperature of the interface and the ion source was 280°C. For these samples, mass spectra were recorded between 35 and 500 amu at 0.30 scan · s^{-1}, and for samples dissolved in methanol and ethanol at 0.30 scan · s^{-1} and above 35–700 amu. The molecules were identified by quadrupole mass spectrometry with the electron impact (EI) mode generated at 70 eV in the scan acquisition mode. The compounds were identified by comparing the mass spectrum with those in the database (Wiley, 9th edition).

The data obtained in the culture pairing test, volatile and non-volatile metabolites were subjected to analysis of variance and the means were compared using the Skott-Knott test at 5% probability of error using the SISVAR 5.3 statistical program (Ferreira 2014).

Results

Molecular and morphological characterization of *Trichoderma* spp.

With the sequencing of the elongation factor region (TEF1-α), it was observed that isolates TR1 and TR4 were allocated to the same clade as other isolates of the species *Trichoderma asperellum*, with a bootstrap of 100 (Fig. 1). Thus, the isolates were identified as *T. asperellum*.

The sporulation between the isolates ranged from 3.85×10^7 (TR1) a 6.31×10^7 (Qt) spores/ml, in which the *T. asperellum* isolate (Qt) showed the highest sporulation among the isolates (Table 2). The final average diameter of the colonies of all *T. asperellum* isolates was 90.00 mm, whereas the mycelial growth rate for TR1 and TR4 isolates was 22.50 mm · day^{-1} and for the Qt isolate it was less than 19.79 mm · day^{-1}. There was variation in the size of the conidia, with an average of 3.51 (*T. asperellum* – Qt) to 3.85 (*T. asperellum* – TR1) µm and width between the conidia 3.19 (*T. asperellum* – TR4) to 3.24 µm (*T. asperellum* – Qt) among the isolates. The colonies had green tones for all isolates of *T. asperellum* (TR1, TR4 and Qt).

Direct confrontation

Regarding the mycelial growth of *L. theobromae* (S6) and *P. kimberleyense* (SM21), there was a significant difference between treatments with the antagonist, in relation to the control (Table 3). However, there was no statistical difference between the isolates of *T. asperellum* (TR1, TR4 and Qt). The control treatment of *L. theobromae* (S6) was the one with the largest diameter of the colony on the fifth day of growth, occupying the entire surface of the medium and differing statistically from *P. kimberleyense* (SM 21).

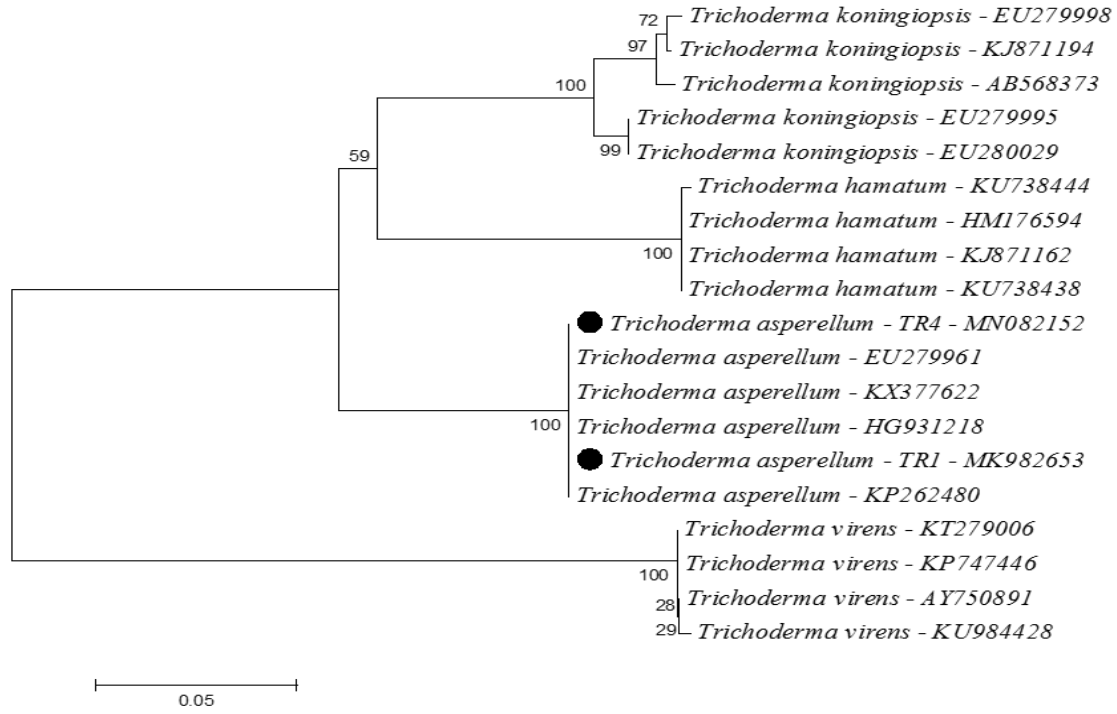

Fig. 1. Phylogenetic dendrogram based on the Neighbor-joining method, obtained from DNA sequences from the elongation factor 1-α region

Table 2. Morphological and physiological characteristics of *Trichoderma asperellum* isolates cultivated on potato dextrose agar (PDA) medium

Isolated	Esp. [10^7]	DC [mm]	T.C.M. [mm · dia^{-1}]	Spore [µm]		Mycelium pigmentation	
				width	length	higher	bottom
TR1	3.85	90.00	22.50	3.29	3.85	grayish green	grayish green
TR4	5.77	90.00	22.50	3.19	3.52	light olive green	light green
Qt	6.31	90.00	19.79	3.24	3.51	light olive green	grayish green

PDA culture medium: Esp. – sporulation at 7 days; DC – final mean diameter of the colony; T.C.M. – mycelial growth rate

Table 3. Mycelial growth and percentage of inhibition of mycelial growth of *Lasiodiplodia theobromae* (S6) and *Pseudofusicoccum kimberleyense* (SM21) compared to *Trichoderma asperellum* (TR1, TR4 and Qt) isolates in the culture pairing test

Treatment	Mycelial growth [mm]		% of inhibition	
	S6	SM21	S6	SM21
TR1	45.55 Ab*	40.67 Ab	43.38 Aa	30.44 Ba
TR4	41.06 Ab	42.61 Ab	48.98 Aa	27.18 Ba
Qt	42.80 Ab	42.44 Ab	46.80 Aa	27.41 Ba
Test	90.00 Aa	58.47 Ba	–	–
CV [%]	12.67		10.84	

*averages followed by the same uppercase letter in the row and lowercase letter in the column do not differ statistically by the Scott-Knott test at 5% probability of error

The inhibition values ranged from 27.41% (TR4 × SM21) to 48.98% (TR4 × S6), in which the greatest reduction in mycelial growth was for *L. theobromae* (S6) compared to *P. kimberleyense* (SM21). In this study, it was also observed that *T. asperellum* isolates did not present statistical differences between them in relation

to *in vitro* control for the same phytopathogenic isolate, mainly due to the fact that these isolates are of the same species, and therefore present similar mycelial growth, as can be seen in the morphological characterization (Table 2).

Volatile metabolites

In the volatile metabolites test, all treatments with *T. asperellum* inhibited the growth of *L. theobromae* (S6) and *P. kimberleyense* (SM21), and there was a significant interaction between the factors (Table 4). On the fourth day after the installation of the experiment, the isolate that showed the greatest mycelial growth was S6 (90.00 mm), which filled the entire surface of the culture medium during this period, while the isolate SM21 grew only 64.59 mm.

As for mycelial growth inhibition, the volatile metabolites produced by *T. asperellum* (TR4), when placed in contact with the *L. theobromae* isolate (S6), presented the lowest percentage of inhibition (7.14%). This result is different from that observed for the isolate *P. kimberleyense* (SM21), since the highest percentage of inhibition of mycelial growth was obtained when it was confronted with the isolate TR4 of *T. asperellum* (29.85%).

Non-volatile metabolites of *Trichoderma asperellum* in the inhibition of mycelial growth of *Lasiodiplodia theobromae* and *Pseudofusicoccum kimberleyense*

In reducing the mycelial growth of *L. theobromae*, in general, the best results came from the culture filtrates obtained through condition eight of development of *T. asperellum* strains, with the cultivation variables being: pH 5, agitation 100 rpm, sucrose 50 g · l^{-1}, inoculum concentration 10^5 spores/ml (Table 5). The greatest reduction was achieved by the filtrate of isolate TR1, which was statistically different from the others and reduced the mycelial growth of *L. theobromae* by 49.12%.

For the filtrates obtained from the *T. asperellum* isolate (Qt), conditions two and three also showed a reduction in mycelial growth above 40.00%. This result was even higher than for condition eight. For the isolate of *T. asperellum* (TR1), a reduction in mycelial growth above 40.00% was observed for conditions five and six, and for the isolate of *T. asperellum* (TR4) for condition five, that is, the culture condition to obtain the filtrate with the greatest antifungal effect varied according to the isolate. Thus, condition eight can be chosen as the best, as it shows greater stability in the production of filtrates with an antifungal effect.

For *P. kimberleyense* (SM21), condition eight again stands out as the one that most reduced the mycelial growth of the pathogen *in vitro* (Table 6). When the culture filtrates obtained from the TR1 strain of *T. asperellum* were evaluated, condition eight (pH 5, agitation 100 rpm, sucrose 50 g · l^{-1}, inoculum concentration 10^5 spores/ml) was the only condition that generated filtrates with antifungal action. This pathogen showed lower sensitivity to culture filtrates when compared to *L. theobromae*, and the only one with an inhibition percentage above 40.00% was the filtrate obtained from condition eight of the cultivation of the Qt strain of *T. asperellum*.

Chromatographic analysis of the culture filtrate of *Trichoderma asperellum* isolates

The best condition for the production of *T. asperellum* filtrates with inhibitory action on the mycelial growth of *L. theobromae* and *P. kimberleyense* was eight (pH 5, 100 rpm agitation, 50 g · l^{-1} of sucrose and 10^5 conidia · ml^{-1}). Thus, the identification of the compounds present in this culture filtrate was carried out by means of gas chromatography. Table 7 shows the compounds extracted by the solvents used in liquid-liquid extraction. Each solvent extracts different compounds, mainly due to differences in polarity between them.

Table 4. Mycelial growth and percentage of mycelial growth inhibition *Lasiodiplodia theobromae* (S6) and *Pseudofusicoccum kimberleyense* (SM21), grown in the presence of volatile metabolites of *Trichoderma asperellum* isolates (TR1, TR4 and Qt), *in vitro*

Treatments	Mycelial growth [mm]		% of inhibition	
	S6	SM21	S6	SM21
TR1	65.44 Ac*	53.25 Bb	27.29 Aa	17.56 Bb
TR4	83.57 Ab	45.31 Bc	7.14 Bb	29.85 Aa
Qt	77.57 Ab	54.18 Bb	13.81 Ab	16.11 Ab
Test	90.00 Aa	64.59 Ba	–	–
CV [%]	6.85		20.76	

*averages followed by the same lowercase letter in the column and uppercase in the row do not differ significantly from each other, at 5% probability of error, by the Skott-Knott Test. Where: CV (%) = coefficient of variation

Table 5. Effect of culture filtrates of different *Trichoderma asperellum* strains (TR1, TR4 and Qt) on mycelial growth (mm) and on the percentage of inhibition of mycelial growth (%) of *Lasiodiplodia theobromae* (S6)

Trat	pH	Agitation [rpm]	Sucrose [g· l^{-1}]	Spores [ ]**	Mycelial growth [mm]			Inhibition [%]		
					TR1	TR4	Qt	TR1	TR4	Qt
1	7	100	50	10^7	63.84 Bd*	90.00 Aa	52.39 Ce	29.07 Bb	0.00 Cd	41.79 Ab
2	7	180	50	10^5	66.77 Bd	90.00 Aa	47.40 Ce	25.81 Bb	0.00 Cd	47.33 Aa
3	7	180	100	10^5	90.00 Aa	90.00 Aa	47.34 Be	0.00 Bd	0.00 Bd	47.40 Aa
4	5	180	100	10^7	90.00 Aa	90.00 Aa	88.06 Aa	0.00 Bd	0.00 Bd	2.16 Af
5	7	100	100	10^7	49.82 Cc	52.97 Bd	71.19 Ac	44.64 Aa	41.14 Aa	20.90 Bd
6	5	180	50	10^7	48.54 Bc	75.18 Ab	77.50 Ab	46.07 Aa	16.47 Bc	13.89 Be
7	5	100	100	10^5	81.12 Cb	59.11 Bc	86.09 Aa	9.87 Bc	34.32 Ab	4.34 Cf
8	5	100	50	10^5	45.79 Bd	51.48 Ad	50.66 Ae	49.12 Aa	42.80 Ba	43.71 Bb
9	6	140	75	10^6	90.00 Aa	90.00 Aa	64.00 Bd	0.00 Bd	0.00 Bd	28.89 Ac
10	6	140	75	10^6	90.00 Aa	90.00 Aa	65.79 Bd	0.00 Bd	0.00 Bd	26.90 Ac
11	6	140	75	10^6	90.00 Aa	90.00 Aa	63.53 Bd	0.00 Bd	0.00 Bd	29.41 Ac
Test					90.00 Aa	90.00 Aa	90.00 Aa		–	
CV [%]						8.50			13.15	

*averages followed by the same uppercase letter in the row and lowercase letter in the column do not differ statistically by the Scott-Knott test at 5% probability of error;
**[ ] – spore concentration

Table 6. Effect of culture filtrates of different strains of Trichoderma asperellum (TR1, TR4 and Qt) on mycelial growth (mm) and on the percentage of inhibition of mycelial growth (%) of Pseudofusicoccum kimberleyense (SM21)

Trat	pH	Agitation [rpm]	Sucrose [g· l^{-1}]	Spores [ ]**	Mycelial growth [mm]			Inhibition [%]		
					TR1	TR4	Qt	TR1	TR4	Qt
1	7	100	50	10^7	90.00 Aa*	87.54 Aa	73.71 Bb	0.00 Bb	0.00 Bc	15.79 Ab
2	7	180	50	10^7	90.00 Aa	90.00 Aa	78.75 Bb	0.00 Bb	0.00 Bc	10.03 Ac
3	7	180	100	10^5	90.00 Aa	82.22 Ba	90.00 Aa	0.00 Bb	6.06 Ab	0.00 Bd
4	5	180	100	10^7	90.00 Aa	90.00 Aa	90.00 Aa	0.00 Ab	0.00 Ac	0.00 Ad
5	7	100	100	10^7	90.00 Aa	90.00 Aa	90.00 Aa	0.00 Ab	0.00 Ac	0.00 Ad
6	5	180	50	10^7	90.00 Aa	90.00 Aa	90.00 Aa	0.00 Ab	0.00 Ac	0.00 Ad
7	5	100	100	10^5	90.00 Aa	79.94 Bb	90.00 Aa	0.00 Bb	8.67 Ab	0.00 Bd
8	5	100	50	10^5	79.71 Ab	76.23 Ab	45.82 Bc	8.93 Ca	12.90 Ba	47.65 Aa
9	6	140	75	10^6	90.00 Aa	88.33 Aa	90.00 Aa	0.00 Ab	0.00 Ac	0.00 Ad
10	6	140	75	10^6	90.00 Aa	89.21 Aa	90.00 Aa	0.00 Ab	0.00 Ac	0.00 Ad
11	6	140	75	10^6	90.00 Aa	89.61 Aa	90.00 Aa	0.00 Ab	0.00 Ac	0.00 Ad
Test					87.53 Aa	87.53 Aa	87.53 Aa		–	
CV [%]						5.66			20.27	

*Averages followed by the same uppercase letter in the row and lowercase letter in the column do not differ statistically by the Scott-Knott test at 5% probability of error;
**[ ] – spore concentration

Discussion

In this work, isolates of *Trichoderma* spp. from a pecan orchard were used which were characterized morphologically and molecularly. Furthermore, different mechanisms by which *T. asperellum* isolates (TR1, TR4 and Qt) can act to control phytopathogenic fungi were addressed, and different tests were carried out to this end.

The results of the present work are positive, since it was possible to identify with reliability the species of *Trichoderma* using only the sequencing of the elongation factor. Lazarotto *et al*. (2016), using the elongation factor, also identified species of *Trichoderma* isolates, which were allocated in clades with *T. atroviride*, with

Table 7. Identification and percentage of the compounds present in the culture filtrates of *Trichoderma asperellum* extracted with solvents ethanol, methanol and hexane

Nomenclature Iuapac	Ethanol		Methanol		Hexane	
	RT [min]	area [%]	RT [min]	area [%]	RT [min]	area [%]
Isolate TR1						
Hydroxyls						
1,3-Propanediol, 2-(hydroxymethyl)-2-nitro-	5.055	8.390	–	–	–	–
2,7-dimethyl-4,5-octandiol	12.293	4.160	–	–	–	–
Fatty acids						
Hexadecanoicacid, 2-hydroxy-1-(hydroxymeth	21.893	21.77	23.514	19.510	–	–
9-Octadecenamide	24.094	4.460	–	–	–	–
Pyran derivatives and biologically active compounds						
4H-Pyran-4-one, 2-hydroxy-3-methyl-	6.957	11.432	–	–	–	–
Propanoicacid, silver(1+) salt	–	–	5.224	5.210	–	–
Isolate TR4						
Fatty acids						
Hexadecanoicacid, 2-hydroxy-1-(hydroxymeth	21.896	5.579	16.564	11.87	–	–
Octadecanoicacid, 2-hydroxy-1-(hydroxymethyl	21.802	8.491	–	–	–	–
9-Octadecenamide	22.139	3.240	–	–	–	–
Pentadecanoicacid	17.479	5.173	–	–	–	–
11-Octadecenoic acid, methylester,	–	–	18.302	2.870	–	–
Hexatriacontane	–	–	–	–	21.668	44.771
Pyran derivatives and biologically active compounds						
4H-Pyran-4-one, 2-hydroxy-3-methyl-	6.190	7.080	–	–	–	–
2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4	7.161	4.930	7.160	5.201	–	–
Propanoicacid, silver(1+) salt	–	–	5.049	3.010	–	–
Celidoniol, deoxy	–	–	40.288	6.223	–	–
Isolate Qt						
Hydroxyls						
DL-Methyltartronic acid	3.055	2.251	–	–	–	–
2,4(1H,3H)-Pyrimidinedione, 5-methyl-	–	–	6.183	4.602	–	–
Guanosine	–	–	12.294	6.641	–	–
Fatty acids						
Hexadecanoicacid, 2-hydroxy-1-(hydroxymeth	23.514	3.020	–	–	–	–
Octadecanoicacid, 2-hydroxy-1-(hydroxymethyl	18.300	1.851	–	–	–	–
9-Octadecenamide	24.082	4.390	–	–	–	–
Hexadecanoicacid, methylester	–	–	16.564	10.560	–	–
11-Octadecenoic acid, methylester,	–	–	23.504	3.552	–	–
Pyran derivatives and biologically active compounds						
4H-Pyran-4-one, 2-hydroxy-3-methyl-	6.211	7.510	–	–	–	–
2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4	7.161	2.558	–	–	–	–
Propanoicacid, 1-methylethyl ester	–	–	–	–	7.413	610

a bootstrap of up to 99. According to Samuels (2006), the elongation factor is efficient in identifying *Trichoderma* species, as it is a region with greater variation than the ITS (Internal Transcribed Spacer), better reflecting the differences between species and intraspecific.

The colorations presented by the isolates used in the present study are similar to those described by Singh and Sharma (2020). These authors, when evaluating 15 different isolates of *Trichoderma*, reported colors that varied from shades of white, light green to dark green, with rapid mycelial growth in PDA culture medium. It is important to note that the morphological characteristics of the isolates of *Trichoderma* spp. are more linked to the nature of the microorganism than to the culture conditions (Milan *et al*. 2015)

After identifying the isolates, the direct confrontation test was carried out. In this, microorganisms compete for nutrients and space, where the one that is faster in using the available resources, multiplies and quickly colonizes the cultivation medium, standing out over the others. This process is similar to what occurs in the soil, when Trichoderma quickly colonizes the rhizosphere, preventing the pathogenic microorganism from attacking the plant.

For inhibition values ranged from 27.41% (TR4 × SM21) to 48.98% (TR4 × S6), where the largest reduction in mycelial growth was for *L. theobromae* (S6) Comparing to *P. kimberleyense* (SM21). This fact is explained by the mycelial growth of *L. theobromae* which was greater in the same period of time, and therefore parasitism between it and *T. asperellum* isolates was occurring more markedly at the time of Theevaluation of the experiment, It is while for *P. kimberleyense*, the main mechanism that was acting was still antibiosis

The *T. asperellum* isolates showed no statistical difference between them with respect to in vitro control for the same phytopathogenic isolate, mainly due to the fact that these isolates are from the same species, and therefore present similar mycelial growth. When grown in liquid medium, *T. asperellum* isolates produced greater dry biomass in treatments with a greater amount of sucrose added to the medium. This is due to the fact that sucrose is a source of energy for fungal development. The liquid medium also allowed *T. asperellum* to produce chitinase, an important enzyme released by fungi of this genus. In this same experiment, the antagonist isolates inhibited the mycelial growth of the stem canker causing isolates subjected to the culture pairing test. However, there was no statistical difference between isolates of *T. asperellum*, only between *L. theobromae* (S6) and *P. kimberleyense* (SM21).

Inhibition similar to the one mentioned above was described by Tapwai and Pandey (2016), with another fungus from the Botryosphaeriaceae family (*Botryodiplodia palmarum*), and the volatile compounds released by different *Trichoderma* species restricted the pathogen's growth in the range of 12.59–22.22%. Although the inhibition is lower, the main advantage of antibiosis, in the case of volatile compounds, is that the toxic substances released by the antagonists can diffuse through the pores present in the soil, inhibiting the development of the pathogen without physical contact with it.

During the growth of *T. asperellum* isolates in liquid culture medium, nutrients were used for their development and released secondary metabolites, responsible for inhibiting the growth of pathogens observed in this research. In similar experiments, Mishra *et al*. (2011) observed that non-volatile compounds, obtained from culture filtrates of *Trichoderma viride*, inhibited the growth of *Rhizoctonia solani*, *Colletotrichum capsici*, *Sclerotinia rolfsii* and *Macrophomina phaseolina*. On the other hand, the culture filtrates obtained from different *Trichoderma* species added to the culture medium of *Botryodiplodia palmarum* inhibited the mycelial growth of the pathogen. At the proportion of 10%, as used in this research, the inhibition ranged from 4.38 to 41.10%, which was similar to the results obtained in the present study (Tapwai and Pandey 2016). In other works, this effect varied according to the fungal species, as in the work carried out by Adebesin *et al*. (2009).

According to Table 7, although the isolates are of the same fungal species, they produced different metabolites during the submerged fermentation process. The same species of *Trichoderma* can produce different compounds and, on the other hand, different species can originate the same compound. This difference in the production of compounds is directly related to the microorganism, the environment (pH and temperature) and the substrate on which the culture is carried out, and this also interferes with the percentage of inhibition found with the use of culture filtrates (Sanchez *et al*. 2019).

According to Resende *et al*. (2003), hydroxyls are active oxygen species that cause a defense response in plants when recognizing a pathogenic attack, activating the plant's immune signaling system. According Pohl *et al*. (2011) fatty acids have antimicrobial action on fungi, mainly on the cell membrane, increasing the fluidity of the membrane, resulting in generalized disorganization, which leads to conformational changes and extravasation of intracellular components.

Among the extracted compounds produced by *T. asperellum* isolates, some stand out for having known bioactive action. Among them are hexadecanoic acid and alpha-humulene, described as insecticides, while others have antimicrobial action, such as pyran derivatives, celidoniol, deoxy, pentadecanoic acid, 2,3-dihydro-3,5-dihydroxy-6- methyl, propanoic acid, 1-methylethyl ester and 9-octadecenoic acid (Ali *et al*. 2017; Barretto and Vootla 2018; Chhouk *et al*. 2018).

Despite the identification of bioactive compounds in the samples, most of the metabolites (greater than 40%) are composed of 2-furancarboxaldehyde, 5-(hydroxymethyl). This compound is a furan derived from the degradation of sugars, in the case of the present research of sucrose. This data is not shown in the table, as it is a compound of the culture medium and not the metabolism of the fungus. Jöbstl *et al*. (2010) commented that the metabolite is formed by reductions and degradations catalyzed by acids, which may have occurred during fungal growth or during the storage of samples. This demonstrates that during the cultivation of *T. asperellum*, not all the sucrose used in the medium was used in fungal development, which is a factor that explains the variability in the results of the inhibition of mycelial growth of pathogens.

Conclusions

Trichoderma asperellum isolates decreased mycelial growth of *L. theobromae* and *P. kimberleyense* in the dual culture test, as well as by non-volatile metabolites. Of the growth conditions tested, the one that generated culture filtrates with the greatest fungicidal potential was eight: 20 $g \cdot l^{-1}$ of corn maceration water (AMM), yeast extract 7.5 $g \cdot l^{-1}$, pH 5, agitation 100 rpm, sucrose 50 $g \cdot l^{-1}$ and an inoculum concentration of 10^5 spores $\cdot$ ml^{-1}. *Trichoderma asperellum* isolates, when cultivated in liquid medium, produced compounds with fungicidal potential. Understanding the biocontrol mechanisms with which *T. asperellum* isolates act on phytopathogenic agents leads to a more efficient application of these biological control agents. In view of this, the biocontrol mechanisms are complex and their synergistic action resulted in the control of pathogens *in vitro*, which can be recommended for *in vivo* research.

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References

Adebesin A.A., Odebode C.A., Ayodele A.M. 2009. Control of postharvest rots of banana fruits by conidia and culture filtrates of *Trichoderma asperellum*. Journal Plant Protection Research 49 (3): 302–308. DOI: https://doi.org/10.2478/v10045-009-0049-6

Ali A., Javaid A., Shoaib A. 2017. GC-MS analysis and antifungal activity of methanolic root extract of *Chenopodium album* against *Sclerotium rolfsii*. Planta Daninha 35: 1–8. DOI: https://doi.org/10.1590/s0100-83582017350100046

Altschul S.F., Madden T.L., Schäffer A.A., Zhang J., Miller W., Lipman D.J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Research 25 (17): 389–402. DOI: https://doi.org/10.1093/nar/25.17.3389

Barretto D., Vootla S. 2018. GC-MS analysis of bioactive compounds and antimicrobial activity of *Cryptococcus rajasthanensis* ky627764 isolated from bombyx mori gut microflora. International Journal of Advanced Research 6 (3): 525–538. DOI: https://doi.org/10.21474/IJAR01/6700

Chen F., Zheng X., Zhao X., Chen F. 2019a. First Report of *Lasiodiplodia theobromae* Causing Stem Canker of *Fraxinus americana*. Plant Disease 103 (12). DOI: https://doi.org/10.1094/PDIS-04-19-0892-PDN.

Chen L., Wang Z., Zhang B., Ge M., Ng H., Niu Y., Liu L. 2019b. Production, structure and morphology of exopolysaccharides yielded by submerged fermentation of *Antrodia cinnamomea*. Carbohydr Polymer, 205 (1): 271-278. DOI: https://doi.org/10.1016/j.carbpol.2018.10.070.

Chhouk K., Wahyudiono H., Goto M. 2018. Efficacy of supercritical carbon dioxide integrated hydrothermal extraction of Khmer medicinal plants with potential pharmaceutical activity. Journal of Environmental Chemical Engineering 6: 2944–2956. DOI: https://doi.org/10.1016/j.jece.2018.04.036

Dennis C., Webster J. 1971. Antagonistic properties of species-groups of *Trichoderma*. Transactions of the British Mycological Society 57 (1): 41–48. DOI: https://doi.org/10.1016/S0007-1536(71)80078-5

Ferreira D.F. 2014. Sisvar: a guide for its bootstrap procedures in multiple comparisons. Ciência e Agrotecnologia 38 (2): 1–4. DOI: https://doi.org/10.1590/S1413-70542014000200001

Isaias C.O., Martins I., Silva J.B.T., Silva J.P., Melo S.C.M. 2014. Ação antagônica e de metabólitos bioativos de *Trichoderma* spp. contra os patógenos *Sclerotium rolfsii* e *Verticillium dahliae*. Summa Phytopathologica 40 (1): 1–8. DOI: https://doi.org/10.1590/S0100-54052014000100005

Junges E., Muniz M.F.B., Campos A.D., Brun T., Michelon C.J., Mazutti M.A. 2018. Bioproducts from *Trichoderma harzianum* as inducer of resistance to anthracnose in beans. p. 70–80. In: "Avanços Científicos e Tecnológicos em Bioprocessos" Organizador Alberdan Silva Santos. Ponta Grossa (PR): Atena Editora.

Jöbstl D., Husøy T., Alexander J. 2010. Analysis of 5-hydroxymethyl-2-furoic acid (HMFA) the main metabolite of alimentary 5-hydroxymethyl-2-furfural (HMF) with HPLC and GC in urine. Food Chemistry 123 (3): 814–818. DOI: https://doi.org/10.1016/j.foodchem.2010.05.015

Lazarotto M., Oliveira L da S., Harakava R., Zanatta P., Farias C.R.J. 2016. Identificação de fungos emboloradores em madeira de *Pinus* spp. em laboratório. Floresta e Ambiente 23 (4): 602–605. DOI: https://doi.org/10.1590/2179-8087.144615

Milan M.D., Barroso F.M., Melo S.C.M., Araújo M.S. 2015. Light regimes used for producing *Trichoderma harzianum* conidia to control white mold in common bean plants. Pesquisa Agropecuária Tropical 45 (4): 434–439. DOI: https://doi.org/10.1590/1983-40632015v4537755

Mishra B.K., Mishra R.K., Mishra R.C., Tiwari A.K., Ydav R.S., Dikshit A. 2011. Biocontrol efficacy of *Trichoderma viride* isolates against fungal plant pathogens causing disease in *Vigna radiata* L. Archives of Applied Science Research 3 (2): 361–369.

Munsell Color. Munsell Soil Color Charts. Munsell Color. 2009. [Available on: http://munsell.com/color-products/color-communications-products/environmental-color-communication/munsell-soil-color-charts/]. [Accessed: Jun 4, 2023]

Perrone G., Stea G., Epifani F., Varga J., Frisvad J.C., Samson R.A. 2011. *Aspergillus niger* contains the cryptic phylogenetic species *A. awamori*. Fungal Biology 115 (11): 38–50. DOI: https://doi.org/10.1016/j.funbio.2011.07.008

Pohl C.H., Kock J.L.F., Thibane V.S. 2011. Antifungal free fatty acids: A review. p. 61–71. In: "Science against Microbial Pathogens: Communicating Current Research and Technological Advances" (Mendez-Vilas A., ed.). 1st ed. Formatex.

Poletto T., Maciel C.G., Muniz M.B., Blume E., Poletto I., Brioso P. 2016. First report of stem canker caused by *Lasiodiplodia subglobosa* on *Carya illinoinensis* in Brazil. Plant Disease 100 (5): 1016. DOI: https://doi.org/10.1094/PDIS-08-15-0948-PDN

Resende M.L.V., Salgado S.M.L., Chaves Z.M. 2003. Espécies ativas de oxigênio na resposta de defesa de plantas a patógenos. Fitopatologia Brasileira 28 (2): 123–130. DOI: https://doi.org/10.1590/S0100-41582003000200001

Rolim J.M., Savian L.G., Walker C., Rabuske J.E., Sarzi J.S., Muniz M.F.B., Silva J.C.P da. 2020. First report of stem canker caused by *Neofusicoccum parvum* and *Pseudofusicoccum kimberleyense* on *Carya illinoinensis* in Brazil. Plant Disease 104 (11): 3067. DOI: https://doi.org/10.1094/PDIS-01-20-0082-PDN

Rolim J.M., Rabuske J.E., Savian L.G., Walker C., Sarzi J.S., Silva J.C.P., Muniz MB. 2022. Fungi of the botryosphaeriaceae family cause different levels of stem canker on pecan trees (*Carya illinoinensis*) in Brazil. Revista Árvore 46 (6): e4615. DOI: https://doi.org/10.1590/1806-908820220000015

Samuels G.J. 2006. *Trichoderma*: Systematics, the sexual state, and ecology. Phytopathology 96 (2): 195–206. DOI: https://doi.org/10.1094/PHYTO-96-0195

Sanchez A.D., Ousset M.J., Sosa M.C. 2019. Biological control of *Phytophthora collar* rot of pear using regional *Trichoderma* strains with multiple mechanisms. Biological Control 135: 124-134. DOI: https://doi.org/10.1016/j.biocontrol.2019.05.012

Singh P., Sharma M. 2020. Cultural and morphological characterization of antagonistic *Trichoderma* isolates. International Journal of Current Microbiology and Applied Sciences 9 (3): 1041–1048. DOI: https://doi.org/10.20546/ijcmas.2020.903.122

Tamura K., Dudley J., Nei M., Kumar S. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) Software Version 4.0. Molecular Biology and Evolution 24 (8): 1596–1599. DOI: https://doi.org/10.1093/molbev/msm092

Tapwai A., Pandey H. 2016. *In vitro* evaluation of *Trichoderma* species for virulence efficacy on *Botryodiplodia palmarum*. Current Life Sciences 2 (3): 86–91. DOI: http://dx.doi.org/10.5281/zenodo.61073

Thompson J.D., Higgins D.G., Gibson T.J., Clustal W. 1994. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22 (22): 4673–4680. DOI: https://doi.org/10.1093/nar/22.22.4673

Vázquez 2016. Retos y opurtunidades para el aprovechamiento de la Nuez pecanera in México. Centro de Investigacíon y Asistencia in Tecnología y Diseno del Estado de Jalisco, A.C. (CIATEJ), 124 pp.

Vinale F., Sivasithamparam F., Ghisalberti E.L., Woo S.L., Nigro M., Marra R., Pascale A., Ruocco M., Lanzuize S., Manganiello G., Lorito M. 2014. *Trichoderma* Secondary Metabolites Active on Plants and Fungal Pathogens. The Open Mycology Journal 8: 127-139. DOI: https://doi.org/10.2174/1874437001408010127