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IN VITRO BIOCONTROL OF *LASIODIPLODIA THEOBROMAE* BY ISOLATES OF *TRICHODERMA* SPP.

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ABSTRACT: Biological control has commonly been established as an alternative to the use of chemical products to control pests and diseases. Fungi of the genus *Trichoderma* are of great economic importance for agriculture, as they are capable of acting as disease control agents for various cultivated plants. With the objective of evaluating the antagonistic potential of isolates of *Trichoderma* spp to *Lasiodiplodia theobromae*, the present work was carried out in a completely randomized design with 7 treatments 6 isolates of *Trichoderma* spp + control. The culture pairing method was used and subsequently the rating scale by Bell et al. (1982) being evaluated on the 7th and 14th days, daily measurements of the growth of the pathogen colonies were measured. Mycelial growth, mycelial growth speed index (IVCM) and antagonistic potential were evaluated. The data were subjected to analysis of variance and the means were compared using the 5% Tukey test. There was a significant effect on the growth of the phytopathogen for all *Trichoderma* isolates tested and the 4 best were considered: TC 01, TC 02, TC 03 and TC 04 by assigning grades and by (IVCM), antagonistic or efficient. Thus, the results obtained showed an inhibitory effect of Trichoderma spp isolates on *L. theobromae*. To evaluate the production of volatile metabolites, it was verified that there was no significant effect of the treatments on the mycelial growth of *L. theobromae* given the methodology and the lack of studies related to the same.
Keywords: Biological control; Mycelial growth; Antagonism; Inhibition.

BIOCONTROLE IN VITRO DE *LASIODIPLODIA THEOBROMAE* POR ISOLADOS DE *TRICHODERMA* SPP.

RESUMO: O controle biológico comumente vem sendo constituído como uma alternativa ao uso de produtos químicos para o controle de pragas e doença. Fungos do gênero *Trichoderma* são de grande importância econômica para a agricultura, uma vez que são capazes de atuarem como agentes de controle de doenças de várias plantas cultivadas. Com o objetivo avaliar o potencial antagônico de isolados de *Trichoderma* spp a *Lasiodiplodia theobromae*, o presente trabalho foi realizado em delineamento inteiramente casualizado com 7 tratamentos 6 isolados de *Trichoderma* spp + testemunha. Utilizou-se o método do pareamento de cultura e posterior a escala de notas de Bell et al. (1982) sendo avaliada aos 7° e 14° dias, medidas diárias do crescimento das colônias do patógeno foram aferidas. Foi avaliado o crescimento micelial, índice de velocidade do crescimento micelial (IVCM) e o potencial antagônico. Os dados foram submetidos à análise de variância e as médias comparadas pelo teste de Tukey 5%. Verificou-se efeito significativo no crescimento do fitopatógeno por todos os isolados de *Trichoderma* testados e os 4 melhores foram considerados: TC 01, TC 02, TC 03 e TC 04 pela atribuição de notas e pelo (IVCM), antagônicos ou eficientes. Assim, os resultados obtidos evidenciaram efeito inibidor de isolados de *Trichoderma* spp sobre *L. theobromae*. Para avaliação da produção de metabolitos voláteis, foi verificado que não houve efeito significativo dos tratamentos sobre o crescimento micelial de *L. theobromae* dada a metodologia e a falta estudos relacionados ao mesmo.
Palavras-chave: Controle biológico; Crescimento micelial; Antagonismo; Inibição

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1. INTRODUCTION

Due to the growing concern regarding the use of pesticides and the increasing focus on sustainability in environmental issues, the search for biological control sources for pest and disease management is necessary, although not a widespread practice. Biological control has commonly been considered an alternative to the use of chemical products for pest and disease control (CARSTENS et al., 2018).

According to Collinge et al. (2022), biological control is defined as the action of organisms that keep the population of other organisms considered pests or diseases at a lower level than would occur in their absence.

The use of antagonistic fungi for the control of plant diseases has garnered interest in recent years, mainly due to the growing prospects of control efficiency combined with the reduction of the environmental impact caused by pesticides (CARMONA et al., 2019). Among the antagonistic fungi, the most studied are those of the genus *Trichoderma* (CHEN et al., 2021).

Biological control has been increasingly used as an alternative to the use of chemical products for pest and disease control. The biocontrol capacity of the genus *Trichoderma* was first demonstrated in 1932 by Weindling, who suggested its use in disease control (SPIEGEL; CHET, 1998).

Fungi of the genus *Trichoderma* are of great economic importance to agriculture, as they can act as disease control agents for various cultivated plants. Some strains of these fungi have been receiving considerable attention from research due to their versatility of action. They are capable of producing enzymes that degrade the cell walls of other fungi and also produce antifungal substances (antibiotics), presenting a diversity of survival strategies that make them highly competitive in the environment with an extraordinary capacity for proliferation in the rhizosphere (RESENDE et al., 2004).

Trichoderma can interact with the pathogen in various ways, such as antibiosis (an antagonistic relationship where individuals of one population secrete or expel substances that inhibit or prevent the development of individuals from other species' populations), parasitism competition (eco-interaction between two species where one, the parasite, benefits from the other, the host, causing damage of greater or lesser importance but rarely death), competition (intra- or interspecific eco-interaction that occurs when two or more species require the same limited environmental resource), hypovirulence (a molecular mechanism for reducing fungal damage), predation, or induction of host defense, making it one of the most promising fungi with antagonistic potential (GAUCH, 1996).

On the other hand, fungi of the genus *Lasiodiplodia* sp. are widely distributed geographically, typical of tropical and temperate

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regions, known for their polyphagism, being pathogens of an extensive list of host plants, most of which are tropical fruit-bearing plants. Fungi of this genus can cause different symptoms in infected plants, including die-back, canker, and lesions on different parts of the plant, as well as inciting the death of seedlings and grafts (DOMINGUES et al., 2011).

It is noteworthy that in the control of *L Lasiodiplodia theobromae*, the improper use of pesticides and the increasing aggressiveness of the pathogen are commonly observed. Chemical control alone does not offer protection or curative control when damage is caused by this organism's attack. Therefore, adopting a series of additional measures such as cultural management and biological control is recommended (TAVARES, 1995).

Given the lack of studies related to the biological control of diseases caused by this pathogen, as well as alternative control to the use of pesticides, this work aimed to evaluate the antagonistic potential of *Trichoderma* spp. isolates against *L. theobromae*.

2. MATERIAL AND METHODS

The study was conducted in the municipality of Alta Floresta, in the far north of the state of Mato Grosso, 830 km from the capital Cuiabá, at the geographical coordinates 09º 52' 32" South Latitude and 56º 05' 10" West Longitude (Figure 1).

The climate is classified according to Köppen as Awi, meaning tropical wet, with high rainfall during the summer, sometimes exceeding averages of 2,750 mm, and a dry winter with predominantly high temperatures, with an annual average around 26ºC (IBGE, 1997).

The predominant soil types in Alta Floresta-MT are Dystrophic Red-Yellow Argisols, with Red-Yellow Latosols and Yellow Latosols occurring as subdominant in most areas (IBGE, 2006). According to the RADAMBRASIL Project (1980), the terrain can be divided into four geomorphological units: the Interplanaltic Depression of Southern Amazonia, the Apiacás-Sucunduri Plateaus, the Dissected Plateau of the Amazon, and the residual plateaus of Northern Mato Grosso. The urban core of Alta Floresta is at an altitude of 340 m above sea level.

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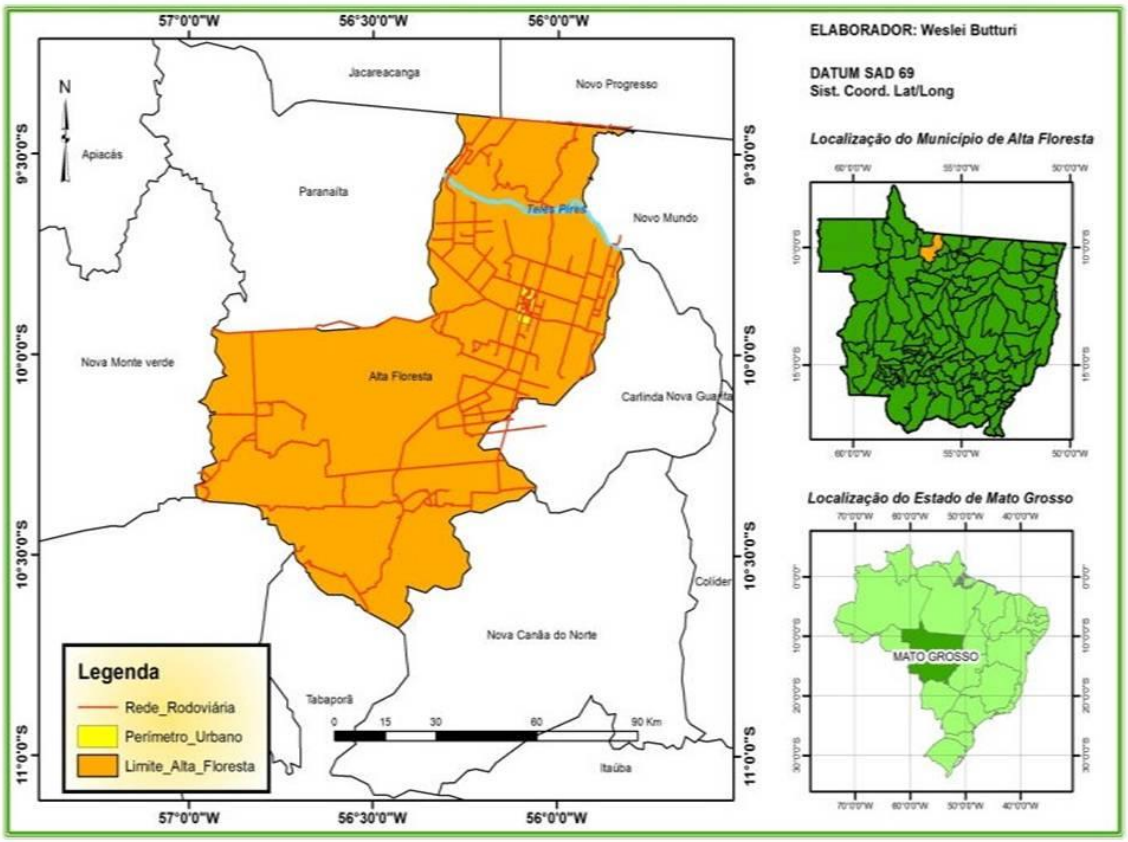
Figure 1 – Map showing the location of the municipality of Alta Floresta – MT.



Source: the authors.

The types of vegetation found in the municipality include tropical open ombrophilous forest, tropical dense forest, savannas, and areas of ecological tension (LOUREIRO et al., 1980). Sánchez (1992) described two eco-regions for the vegetation of northern Mato Grosso: lowland and mountain rainforests and the residual plateaus of northern Mato Grosso (Parecis Plateau).

2.1 METHODOLOGICAL PROCEDURES

The study was conducted in the Microbiology Laboratory at the Universidade do Estado do Mato Grosso – UNEMAT. Four isolates of *Trichoderma* sp. (TC 01, TC 02, TC 03, and TC 04), from the collection of fungi for biological control of phytopathogens at the Microbiology Laboratory's Mycotheque, Unemat, Alta Floresta, and two commercial products based on Trichoderma spp. identified here as commercial product 01 (PC 01) and commercial product 02 (PC 02), were tested for their potential to control *L. theobromae*. All isolates were preserved using the Castellani method (1939), a simple and economical technique using sterile distilled water that ensures the viability, stability, purity, and pathogenicity of a large number of fungal organisms (CASTELLANI, 1967).

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The method involves inoculating small glass vials containing sterilized distilled water with a small portion of culture medium, discs containing mycelium or spores of the fungus to be preserved. These discs can vary in size depending on the size and shape of the glassware (Figure 2).

Figure 2 – *Trichoderma* sp. (TC 01, TC 02, TC 03, and TC 04), along with 2 commercial products (PC01, PC02) belonging to the collection of fungi for the biological control of phytopathogens at the Mycology Collection of the Microbiology Laboratory, Unemat, Alta Floresta.

Source: the authors.

The experimental design used was completely randomized with 7 treatments, each with 15 repetitions. The treatments consisted of 6 *Trichoderma* spp. isolates (TC 01, TC 02, TC 03, and TC 04), from the collection of fungi for biological control of phytopathogens at the Microbiology Laboratory's Mycotheque, Unemat, Alta Floresta, and two commercial products based on Trichoderma spp., identified as commercial product 01 (PC 01) and commercial product 02 (PC 02), plus a control with no addition of antagonist, only the pathogen disc. Each plot consisted of 3 plates.

To evaluate the antagonism of *Trichoderma* isolates against the pathogen, the culture pairing methodology (direct confrontation) described by Dennis and Webster (1971) was used, in which 10 mm Ø discs containing pathogen and antagonist mycelia were placed on opposite sides of Petri dishes (90 mm Ø) at a distance of 0.2 cm from the edge (Figure 3).

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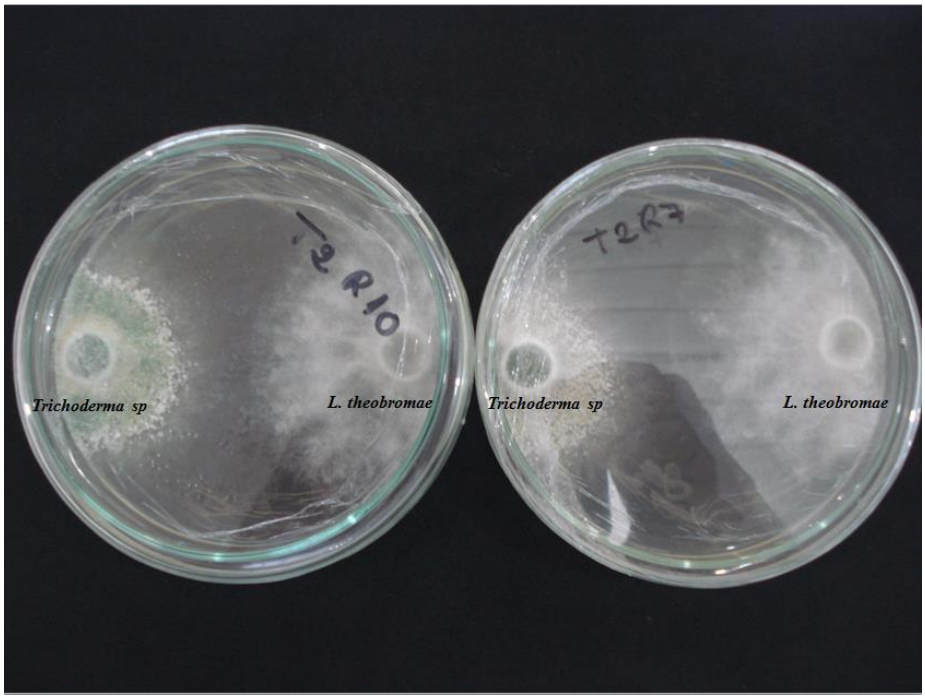
Figure 3 – *Trichoderma* sp. on the right side paired with *Lasiodioplodia theobromae* on the left side.



Source: the authors.

Mycelial growth was estimated on PDA culture medium. The plates were incubated in a BOD growth chamber at a temperature of 27 ºC, with a photoperiod of 12 hours light/dark, for the period it took for the control to fill the plate.

The estimated variables were as follows:

Average mycelial growth: obtained through daily measurements of the colony diameter (mm) of the pathogen;

Mycelial growth rate index (IVCM): obtained from the averages of the daily mycelial growth values of each treatment, as proposed by Oliveira (1991):

$$IVCM = \Sigma(D - Da) / N$$

where,

D = current average diameter of the colony;

Da = average diameter of the colony from the previous day;

N = number of days after inoculation.

The data obtained were subjected to analysis of variance, followed by Tukey's mean comparison test at a 5% significance level, using the Sisvar® program (FERREIRA, 2011).

Volatile metabolites

The assay was conducted in the Microbiology Laboratory at the Universidade do Estado do Mato Grosso – UNEMAT, Alta Floresta campus. The Trichoderma isolates used were four Trichoderma spp. (TC 01, TC 02, TC

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03, and TC 04) from the collection of fungi for the biological control of phytopathogens at the Mycotheque of the Microbiology Laboratory, Unemat, Alta Floresta, and two commercial products based on *Trichoderma* spp., identified as commercial product 01 (PC 01) and commercial product 02 (PC 02). All isolates were preserved using the Castellani method (1939), a simple and economical technique using sterile distilled water that ensures the viability, stability, purity, and pathogenicity of a large number of fungal organisms (CASTELLANI, 1967).

The method involves inoculating small glass vials containing sterilized distilled water with a small portion of culture medium, discs containing mycelium or spores of the fungus to be preserved. These discs can vary in size depending on the size and shape of the glassware.

To verify the inhibition of pathogen growth by volatile metabolites potentially produced by the antagonist, the overlay method was used (BOMFIM et al., 2010). Petri dishes (Ø10 mm) containing PDA medium were positioned one over the other, with a 10 mm PDA disc containing Trichoderma spp. mycelium placed on the lower dish and a disc containing *L. theobromae* placed on the upper dish (Figure 4).

Next, the plates were sealed and incubated in a B.O.D. growth chamber at a temperature of 27 ºC, with a photoperiod of 12 hours light/dark, for the period it took for the control to fill the plate. Each treatment consisted of 10 repetitions in a completely randomized design. For the control, only *L. theobromae* discs were used, both on the lower and upper parts of the Petri dish.

The variables studied included the average mycelial growth, obtained through daily measurements of the pathogen's mycelial growth (mm) in the colonies located on the upper plate using a millimeter ruler. Based on the daily average mycelial growth values for each treatment, the mycelial growth rate index (IVCM) was calculated according to the formula proposed by Oliveira (1991):

$$IVCM = \Sigma(D - Da) / N$$

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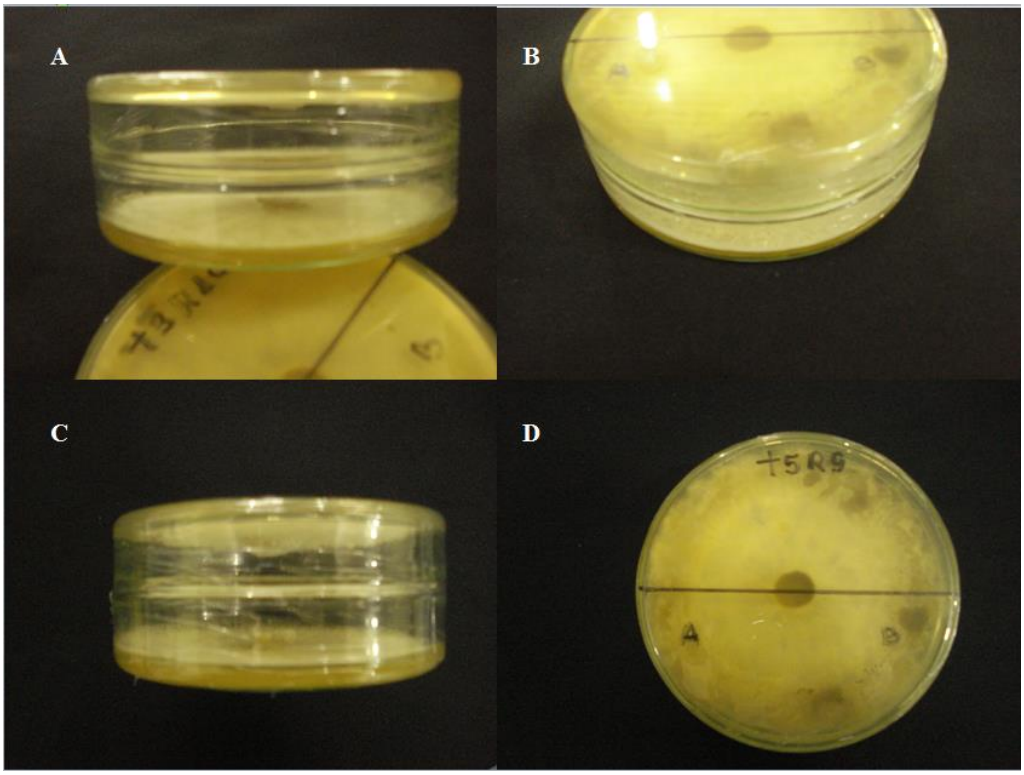
Figure 4 – Letters A, B, C: Scheme for assembling plates; letter D: Evaluation of volatile metabolites through mycelial growth.



Source: the authors.

Bel scale

The antagonistic potential of *Trichoderma* spp. isolates was evaluated at seven and 14 days of paired cultivation using the Bell et al. (1992) scale, with ratings ranging from 1 to 5, where:

1 – Antagonist grows and occupies the entire plate;

2 – Antagonist grows over 2/3 of the plate;

3 – Antagonist and pathogen grow up to half of the plate;

4 – Pathogen grows over 2/3 of the plate;

5 – Pathogen grows over the entire Petri dish.

A score equal to or less than 3.0 was considered indicative of an antagonistic or efficient isolate.

The data obtained were subjected to analysis of variance, followed by Tukey's mean comparison test at a significance level of 5%, using the Sisvar® program (FERREIRA, 2011).

3. RESULTS AND DISCUSSION

All *Trichoderma* isolates inhibited the growth of the phytopathogen. Culture pairing occurred from the third day of evaluation. It was observed that the mean mycelial growth (Table 1) of *L. theobromae*, when paired with *Trichoderma* sp. isolates, showed significant

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differences compared to the control. Similar results, where inhibition of phytopathogen mycelial growth by *Trichoderma* sp. isolates was observed, were obtained by Mello et al. (2007), Martins (2009), Louzada (2009), Bomfin (2010), and Bonett et al. (2013) (Figure 5).

Table 1 – Mycelial growth (3rd and 4th day of evaluation) and mycelial growth rate index (IVCM) of *Lasiodioplodia theobromae* in the presence of commercial products based on Trichoderma spp. and *Trichoderma* spp. isolates evaluated using the pairing technique.

Treatments	Mycelial growth (mm)		IVCM
	Av. 03	Av. 04	
T_1- *Lasiodiplodia theobromae*	45,13 a	62,73 a	18,80 a
T_2- *L. theobromae* **x** PC 01	(*)35,61 b	46,07 b	14,12 b
T_3- *L. theobromae* **x** PC 02	33,11 b	38,53 c	10,09 c
T_4- *L. theobromae* **x** TC 01	30,93 b	33,53 c	6,97 d
T_5- *L. theobromae* **x** TC 02	30,27 b	35,67 c	8,33 d
T_6- *L. theobromae* **x** TC 03	29, 73 b	32,47 c	6,78 d
T_7- *L. theobromae* **x** TC 04	29,66 b	33,20 c	6,18 d
CV%	12,29	7,69	11,6

Means followed by the same letter in the column do not differ significantly from each other according to Tukey's test at a 5% probability level. (*) Diameter of the colony of *L. theobromae* (mm). Source: the authors.

It was observed for the mycelial growth rate index that all treatments differed from the control, indicating inhibition of phytopathogen growth in the presence of *Trichoderma* spp. isolates. Vey, Hoagland, and Butt (2001) reported that this inhibition could be explained by the antagonist's rapid growth, even over the pathogen, likely due to a type of stimulation from the host itself, which is an advantageous characteristic for the antagonist in competing for colonization of the area, thereby outcompeting the pathogen for space or nutrients. Bomfim (2010) inferred that the reduction in the growth of the *Rhizopus* stolonifer phytopathogen colony in the presence of *Trichoderma* spp. could be attributed to the release of metabolites by the antagonists.

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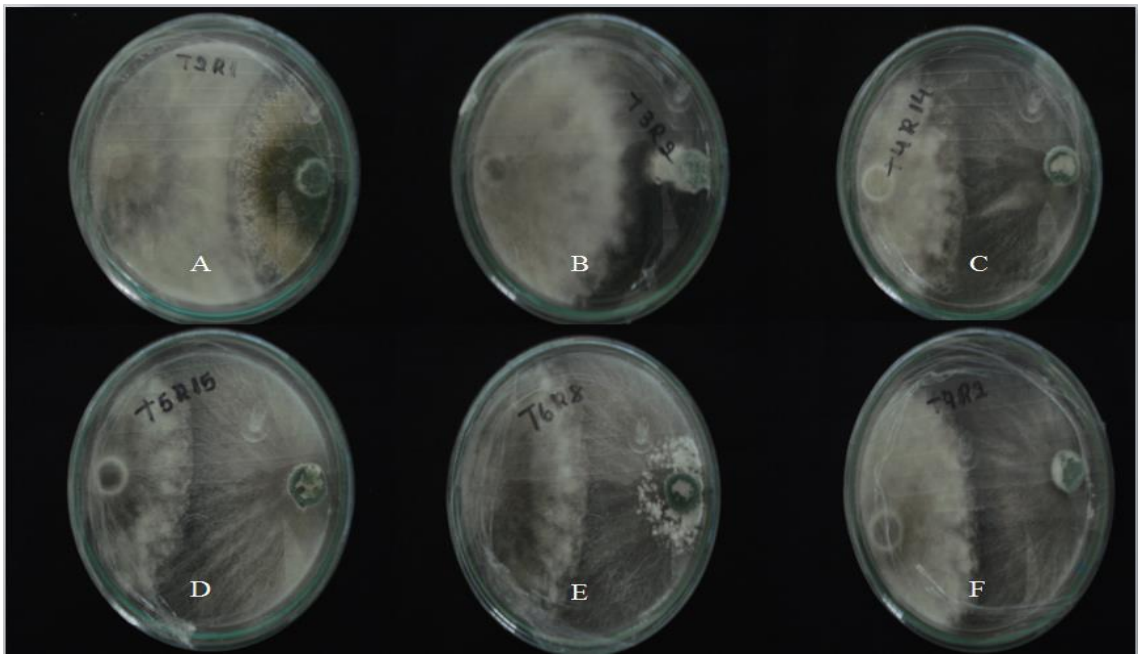
Figure 5 – Letters A, B, C, D, E, F: Trichoderma isolate discs on the right paired with *Lasiodioplodia theobromae* on the left. T2 R1 (PC 01), T3 R2 (PC 02), T4 R14 (TC 01), T5 R15 (TC 02), T6 R8 (TC 03), T7 R2 (TC 04).



Source: the authors.

For the antagonistic potential of *Trichoderma* spp. against *L. theobromae* according to the Bell et al. (1982) scale on the 7th day of paired cultivation, scores equal to or less than 3 were observed for the *Trichoderma* spp. isolates: TC 01, TC 02, TC 03, and TC 04, which was repeated on the 14th day, indicating these isolates as antagonistic or efficient. The other isolates: PC 01 and PC 02, showed moderate antagonistic potential, with scores between 3 and 4 observed for both days (Figure 6).

Louzada et al. (2009) observed scores lower, equal, and higher than 3 for *Trichoderma* isolates tested against *Fusarium solani* and *Sclerotinia sclerotiorum*, suggesting that besides direct parasitism, other mechanisms may be involved in the antagonistic action of *Trichoderma* fungi, such as antibiosis and competition (FRAVEL, 2005).

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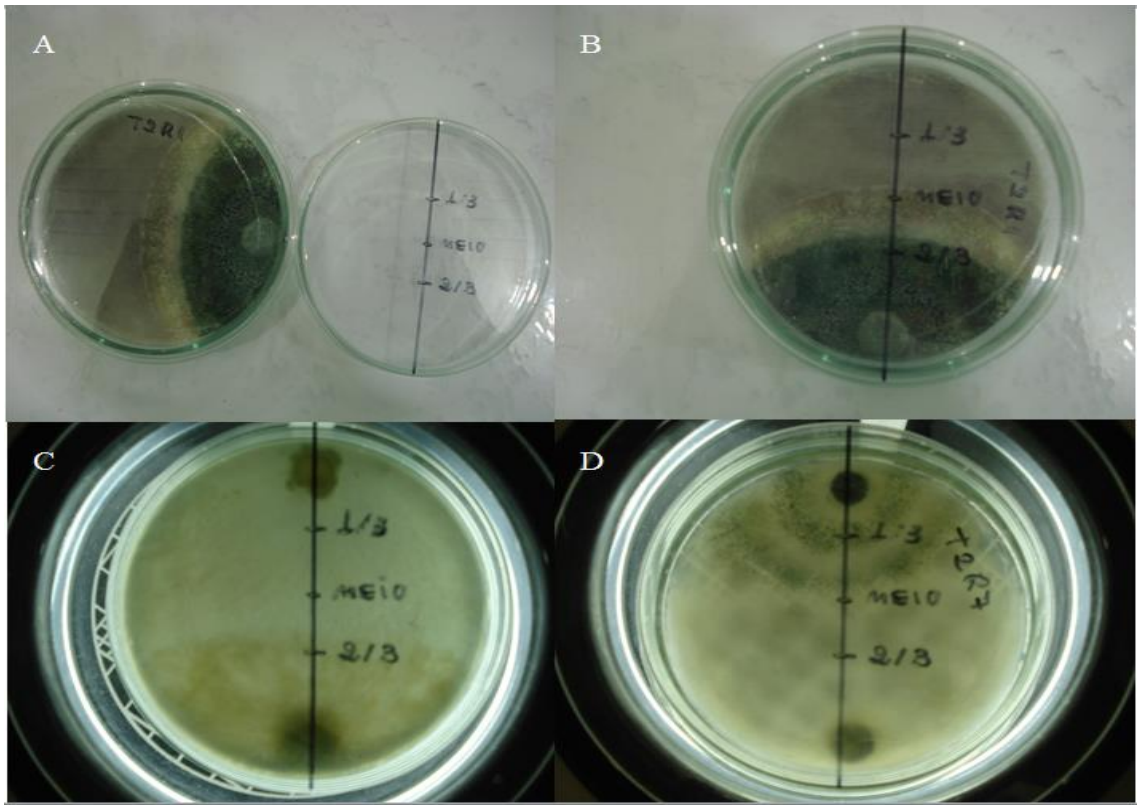
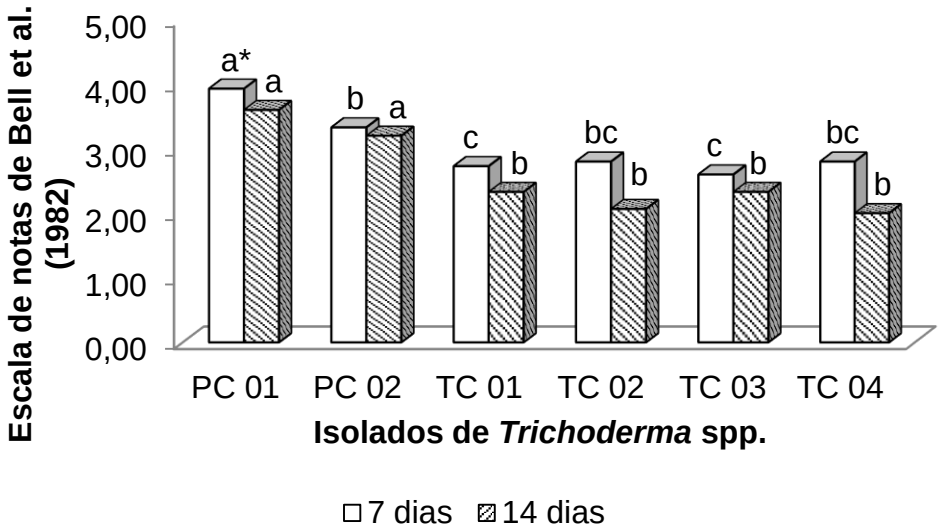
Figure 6 – Letters A, B, C, D: Bell et al. (1982) scale scores assigned to the experiment at the 7th and 14th evaluation days.



Source: the authors.

Figure 7 – Scores assigned to the paired cultivation of *Lasiodiplodia theobromae* with different *Trichoderma* spp. isolates evaluated at 7 and 14 days post-inoculation.



*Means followed by the same lowercase letter for each variable do not differ significantly according to Tukey's test at a 5% probability level. Source: the authors.

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In this study, active mycelial growth and sporulation of *Trichoderma* spp. on *L. theobromae* were observed for the production of volatile metabolites, demonstrating high competitive capacity. Matos et al. (2012) also observed mycelial invasion by T. viride on *L. theobromae*, confirming what was reported by Melo (1996), that some *Trichoderma* strains present a variety of survival strategies that make them highly competitive in the environment.

The results obtained and presented in Table 2 showed that until evaluation 2 (Av. 02), the behavior of the treatments concerning the control was similar. However, greater mycelial growth of the control compared to the other treatments was observed, suggesting a possible inhibition in the mycelial development of *L. theobromae* due to the production of volatile metabolites by the antagonist. In the final evaluation (Av. 03), the lowest mean mycelial growth was observed in treatments T7, T3, and T6, respectively. However, it was found that they did not differ significantly from the other treatments, except for the one that had the highest growth mean (T4). In this case, T4 showed superior mycelial growth compared to the control, indicating no inhibition in mycelial development, but rather a greater growth stimulus, confirmed by the IVCM, with an index of 10.22 (Table 2).

Table 2 – Effect of volatile metabolites produced by *Trichoderma* spp. on the mycelial growth and mycelial growth rate index (IVCM) of *Lasiodiplodia theobromae*. Microbiology Laboratory, UNEMAT.

Treatments	**Micelial growth (mm)**			**IVCM**
	Av. 01	**Av. 02**	**Av. 03**	
T_1- *Lasiodiplodia theobromae*	14,18 a	27,43 a	33,12 ab	8,61 ab
T_2- *L. theobromae* **x** TC 02	(*)13,97 a	26,83 a	32,75 ab	8,41 ab
T_3- *L. theobromae* **x** TC 01	13,42 ab	24,32 ab	30,15 b	7,40 b
T_4- *L. theobromae* **x** PC 01	12,98 ab	26,02 ab	37,14 a	10,22 a
T_5- *L. theobromae* **x** PC 02	12,58 ab	26,37 a	33,17 ab	9,16 ab
T_6- *L. theobromae* **x** TC 03	11,50 ab	24,30 ab	30,35 b	8,42 ab
T_7- *L. theobromae* **x** TC 04	9,70 b	22,37 b	29,20 b	8,61 ab
CV%	15,16	7,39	7,01	12,01

Means followed by the same lowercase letter within the row and uppercase letter within the column do not differ significantly according to Tukey's test at a 5% probability level. (*) Diameter of *L. theobromae* colony (mm). Source: the authors.

It was observed that the control, with *L. theobromae* disks both on the bottom and top of the Petri dish, showed more active mycelial growth on the bottom of the plate, which was

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colonized in a shorter period of time compared to the colony located on the top plate. In this case, the pathogen may have been influenced in its growth due to its position on the plate. In antibiosis tests conducted by Bomfim (2010), the production of volatile metabolites from Trichoderma spp. species was verified, resulting in inhibition of mycelial development of the phytopathogen *Rhizopus* stolonifer. It was also found that the growth of the phytopathogen was reduced in the presence of *Trichoderma* spp., compared to the control, which almost completely covered the plate by the fourth day. Isaias et al. (2014) observed a percentage of mycelial growth inhibition by volatile metabolites of Trichoderma spp. isolates ranging from 40% to 60% for *Sclerotium rolfsii* and inhibitory action close to 80% for *Verticillium dahliae*.

According to Dennis & Webster (1971), *Trichoderma* species are efficient producers of volatile metabolites in culture media. They explain that volatile antibiotics act on susceptible fungi by inhibiting mycelial growth. The results obtained in the present study did not confirm the production of volatile metabolites and the respective control of the phytopathogen by the method used. This does not necessarily mean that their production does not occur, but perhaps the technique is not the most appropriate, given the behavior of T4 on the 1st day, where it showed effective inhibition, and in the subsequent days, there was a decrease in the effect.

Prasad and Kumar (2011) demonstrated that antimicrobial metabolites produced by Trichoderma are effective against a wide range of fungal phytopathogens, such as *Fusarium oxysporum*, *Rhizoctonia solani*, *Curvularia lunata*, *Bipolaris sorokiniana*, *Colletotrichum lagenarium*, *C. acutatum*, and *C. gloeosporioides*. Regarding the effect of volatile metabolites against *L. theobromae*, there are no reports in the literature for further discussion.

4. CONCLUSION

The results obtained from the in vitro culture pairing methodology showed an inhibitory effect of *Trichoderma* spp. isolates on the growth of *Lasiodiplodia theobromae*, representing significant potential for combating phytopathogenic fungi.

The production of metabolites under in vitro conditions revealed a nonsignificant antagonistic action of *Trichoderma* isolates on the growth of *Lasiodiplodia theobromae*, as studies in this area still have few publications, and it is of great importance to conduct more assays for further results.

Studies focused on biological control can significantly contribute to reducing the use of agricultural pesticides and conserving the environment.

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