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CHEMICAL COMPOSITION, ALLELOPATHIC AND ANTIFUNGAL ACTIVITY OF THE CRUDE EXTRACT OBTAINED FROM *TRICHODERMA PSEUDODENSUM*

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ABSTRACT

Fungi of the genus Trichoderma are widely recognized for their biotechnological applications, including biocontrol, plant growth promotion and production of bioactive compounds. In this study, we investigated the biotechnological potential of the fungus Trichoderma pseudodensum, which was incubated in MDB liquid medium (potato and dextrose) for 21 days. After the incubation period, the metabolites produced using ethyl acetate (AcOEt) were extracted to obtain the crude extract. The crude extract was evaluated for antifungal activity against the phytopathogens Curvularia lunata and Sclerotinia sclerotiorum, in addition to an analysis of allelopathic activity in lettuce seeds. The results showed that the extract significantly inhibited the growth of phytopathogens, with emphasis on the inhibition of 74.53% in S. sclerotiorum. Additionally, the allelochemical activity was evidenced by the inhibition of seedling development in 82.8% and root development in 93.7% at the highest concentration tested (1000 mg L^{-1}). For the chemical characterization of the metabolites, the crude extract was analyzed by gas chromatography coupled to mass spectrometry (GC-MS), resulting in the identification of 20 compounds, of which two were majoritarian: 22,23-dibromostigmasterol acetate (11.26%) and di-n-octyl phthalate (44.46%). These results indicate that T. pseudodensum has great biotechnological potential in the production of bioactive compounds, suggesting its use in sustainable agricultural practices.

Keywords: Trichoderma pseudodensum, Filamentous fungi, Bioactive metabolites, Antifungal activity.

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INTRODUCTION

There are 375 known species of the genus Trichoderma (Cai & Druzhinina, 2021). These fungi belong to the division Ascomycota, subdivision Pezizomycotina, class Sordariomycetes, and order Hypocreales (Modrzewska et al., 2022). Members of the Ascomycota family are associated with the presence of individuals that reproduce asexually (anamorph) and individuals that reproduce sexually (teleomorph) (Kubiak et al., 2023).

The fungi of this genus are filamentous and cosmopolitan fungi, they are widely distributed, they occur in all climatic zones, in different environments, such as soil, plants, dead wood and bark. Fungi of Trichoderma species can be easily isolated from the soil, grow rapidly under laboratory conditions, and produce numerous white and green conidia (Modrzewska et al., 2022). They are considered saprophytic organisms, capable of colonizing and decomposing dead organic matter. In addition, they exhibit parasitic properties in relation to other fungi, as well as symbiotic and endophytic abilities in relation to plants (Kubiak et al., 2023).

Trichoderma species have several biotechnological applications. They can act as biocontrol, as biofungicides to protect plants against disease, and as bioremediation agents to clean contaminated environments. In addition, they are involved in the production of enzymes, antibiotics, and other metabolites, which are essential for various industrial processes, including the production of biofuels (Kubiak et al., 2023; Villao-Uzho et al., 2024).

Trichoderma spp. are efficient biocontrol agents that can protect plants against various fungal pathogens. They utilize multiple mechanisms, such as mycoparasitism, competition for nutrients and space, antibiosis, and induction of defense responses in plants (Modrzewska et al., 2022). Biocontrol-efficient Trichoderma strains are being developed as promising biological fungicides. Examples include the strains of T. viride and T. harzianum, which have varying degrees of inhibitory effects on 29 species of plant pathogenic fungi belonging to 18 genera, including Botrytis, Fusarium, and Rhizoctonia (Yao et al., 2023).

Another successful biocontrol agent is T. longibrachiatum T6, which has a good control of sweet pepper damping-off and can effectively control the spread of the disease. The control was up to 54.8%, which is 12.5% higher than that of the chemical pesticide carbendazim. Another example, we can include T. harzianum that demonstrated control over growth by Phytophthora in bell pepper and potato. It can inhibit the growth of

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Phytophthora in the soil, reduce the number of pathogenic fungi, and effectively reduce the rate of dead seedlings and the disease rate in plants (Yao et al., 2023).

Trichoderma species are considered a rich source of distinct bioactive secondary metabolites due to the presence of numerous biosynthetic gene clusters, which allow them to adapt to different ecological biotopes. Several secondary metabolites of Trichoderma have been isolated, including terpenoids, polyketides, peptides, alkaloids, and steroids. Most of these compounds exhibited antimicrobial, cytotoxic, and antifungal effects (Guo et al., 2023; Schmoll & Schuster, 2010). Khan and colleagues reported more than 40 compounds produced by Trichoderma ssp. fungi that exhibited antifungal activity against phytopathogenic fungi (Khan et al., 2020).

The secondary metabolites produced by Trichoderma act as fundamental signaling molecules, allowing it to communicate with the environment and interact with various organisms around it, such as beneficial microorganisms, plants, and pathogens. These metabolites perform varied biological functions during the colonization of the rhizosphere and roots, such as protection from the fungus itself, regulation of plant growth, increased resistance to biotic and abiotic stresses, in addition to limiting the action of bacteria, nematodes, and filamentous pathogens.

In summary, Trichoderma's versatility in terms of biocontrol, synthesis of secondary metabolites, and promotion of plant growth make this genus a huge biotechnological potential in several areas, such as agriculture, biofuels, and pharmaceuticals. In order to evaluate the biotechnological potential of fungi of the genus Trichoderma, the filamentous fungus Trichoderma pseudodensum, was submitted to fermentation using liquid medium and the crude extract ethyl acetate was obtained. The crude extract was submitted to antifungal and allelochemical assays, as well as gas chromatography to identify the secondary metabolites produced by the fungus.

The fungus Trichoderma pseudodensum was first isolated and identified from soil collected in the Shennogjia Nature Reserve in Hubei Province, China (Chen & Zhuang, 2017). Phylogenetically, T. pseudodensum is related to T. zayuense, but they differ in colony morphology and phyalid length. There are no studies on the chemical diversity produced by T. pseudodensum, nor studies of its biotechnological properties.

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MATERIAL AND METHODS

CULTIVATION OF THE FUNGUS IN LIQUID MEDIUM TO OBTAIN THE CRUDE EXTRACT

The filamentous fungus used in the present work was isolated by Alves (2020), and identified by molecular biology as Trichoderma pseudodensum (Alves 2020).

The fungus was grown in Petri dishes containing BDA medium (Potato Dextrose Agar) and incubated in a BOD (Biochemical Oxygen Demand) chamber at a temperature of 25ºC for 7 days. Soon after inoculation in MDB liquid medium (Dextrose and Potato Medium) in 500 mL Erlenmeyers containing 300 mL of culture medium, the Erlenmeyers were kept on bench for 21 days at room temperature.

After this period, the juice was separated from the mycelium by vacuum filtration, using filter paper and Buchner funnel. The filtrate was subjected to liquid/liquid partitioning in triplicate, using chloroform (CHCl3) in a 2:1 ratio. After the chloroform was evaporated in a rotary evaporator under reduced pressure, providing the crude extract AcOEt.

EVALUATION BY GAS CHROMATOGRAPHY WITH MASS SPECTROMETER (CG-MS)

The investigation of the chemical composition of the crude extract was carried out by gas chromatography (GC) analysis coupled to mass spectrometry (EM), at the analytical center of the University of São Paulo (USP), using Shimadzu equipment - Model GCMS-QP202. A large-caliber BP-5 capillary column (30 mm x 0.53 mm i.d., 1.0 mm film thickness) was used. Helium (He) gas was used as a carrier gas, with a flow rate of 1.9 mL min-1 and 107 KPa of inlet pressure. The temperature of the CG furnace was programmed to 0°C to 280°C, with a heating ramp of 2.5 °C min-1. The mass spectrum was obtained in the range of 40-650 amu, operating at 70 eV, and the source was maintained at a temperature of 280°C. The data obtained from the mass spectrum were compared with the NIST mass spectra from the CG-MS database.

ALLEOPATHY TRIAL

The crude extract was subjected to allelopathic activity using lettuce seeds (Lactuca sativa) from the brand Feltrin Sementes. Three different concentrations were used for the assay: 100, 500 and 1000 µg mL-1. The control concentration contained no crude extract and was called concentration 0.

The concentrations were applied in triplicate in Petri dishes containing filter paper. After adding 1 mL of each concentration, 24 hours were waited for the solvent to evaporate

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and 15 lettuce seeds were added to each plate. The seeds were incubated at around 25°C for 5 days. Seeds with 2 mm radicle protrusion were considered germinated. After the 5-day period, the percentage of germinated seeds and root length and total seedlings were evaluated, measured with the aid of a caliper. The test was performed in triplicate and the values of root length and total seedlings were submitted to analysis of variance using the SISVAR 5.7 program and the comparison of means was performed by linear regression with 5% probability.

ANTIFUNGAL EVALUATION

The evaluation of mycelial growth inhibition was performed for the phytopathogenic fungi Curvularia lunata and Sclerotinia sclerotiorum. The test was performed on Petri dishes containing BDA medium. Crude extracts with a concentration of 5 mg mL-1 were added (100 µL) to the surface of the BDA culture medium and spread on the surface of the medium with the aid of a Drigalsky loop. Subsequently, mycelium discs of the phytopathogenic fungus were inoculated in the center of each plate. The witness contained only the phytopathogenic fungus. The test was performed in triplicate. The plates were incubated in BOD, at 25 ºC, for 5 days. The evaluation was carried out by measuring the diameters of the growth colony of the phytopathogenic fungus. The percentage of mycelial growth inhibition was calculated using the formula PIC% = (DT – DTRAT) / DT × 100, where DT is the diameter of the control and DTRAT is the diameter of the colony of each treatment.

RESULTS AND DISCUSSION

CHEMICAL COMPOSITION OF CRUDE EXTRACT

The production of secondary metabolites of the fungus Trichoderma pseudodensum was performed by gas chromatography with mass spectrometer (GC-MS). Figure 2 shows the chromatogram obtained from the analysis.

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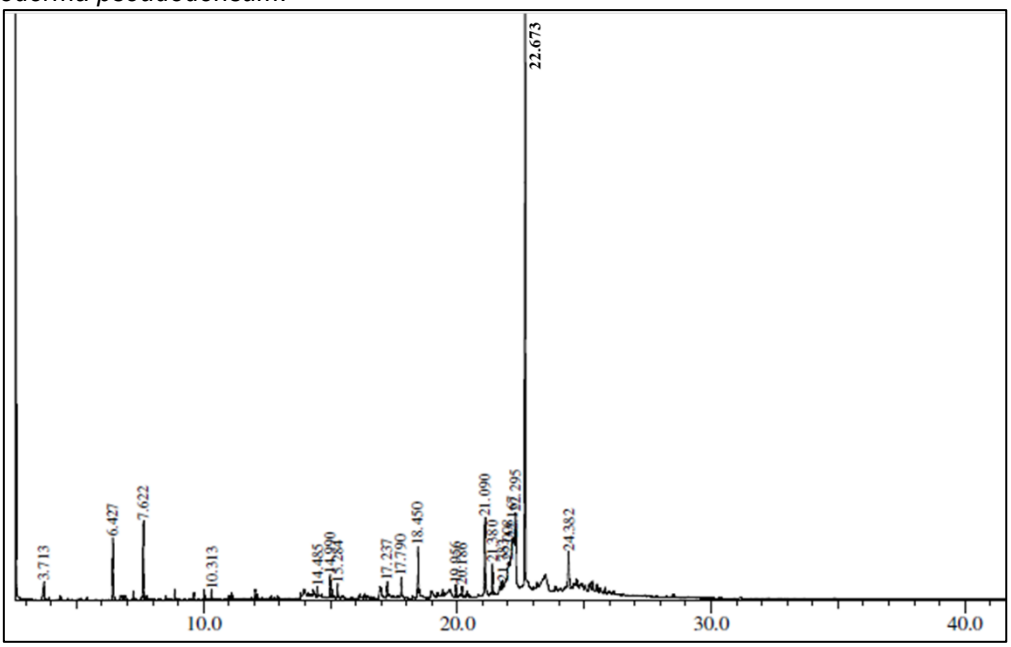
Figure 2 - Chromatogram obtained by GC-MS of the chemical profile of the crude extract obtained from the fungus *Trichoderma pseudodensum*.



Source: The author (2024).

A total of 20 chemical compounds produced by the fungus T. pseudodensum were identified at different retention times and percentage of area (Table 1).

Table 1 - Chemical compounds of the crude extract of the endophyte *Trichoderma pseudodensum*.

Compounds	Name	Tempo right. (min)	% Area	Compound Class
1	4-methyl-2-pentenoic acid	3,713	0,57	Carboxylic acid
2	2-phenylethanol	6,427	3,46	Alcohol
3	(3E, 5E) -nona-3,5-dien-2-ona	7,622	4,66	Unsaturated ketone
4	3-hydroxy-2,2,4-trimethylpentyl 2-methyl-propanoate	10,313	0,57	Ester
5	1,7-dimethyl-4-(1-methylethyl)-spiro[4,5]-dec-6-en-8-ona	14,485	0,65	Ketone
6	Tetrahydroionone	14,990	1,36	Ketone
7	Benzyl benzoate	15,284	0,79	Aromatic ester
8	Palmitic acid	17,237	0,99	Carboxylic acid
9	Bisphenol F	17,790	1,27	Phenol
10	Teephthalato de diisobutila	18,450	2,88	Ester
11	2-(3-hydroxyphenyl) indene-1,3-dione	19,956	0,71	Phenol
12	Tributyl citrate	20,186	0,55	Ester
13	9Z-Octadecenamide	21,090	5,60	Amida
14	dioctyl hexanedioate	21,380	1,90	Ester
15	acetato de (2,4a,5,8a-tetrametil-1,2,3,4,7,8-hexahidronaftaleno-1-il)	21,783	2,51	Ester
16	tris(2-ethylhexyl) phosphate	22,008	7,03	Phosphate
17	acetato de 22,23-dibromostigmasterol	22,167	11,26	Steroid
18	(1E,3Z,6E,10Z,14S)-3,7,11-trimethyl-14-propane-2-ylcyclotetradeca-1,3,6,10-tetraene	22,295	6,08	Diterpene
19	Ftalato de di-n-octilo	22,673	44,46	Phthalate

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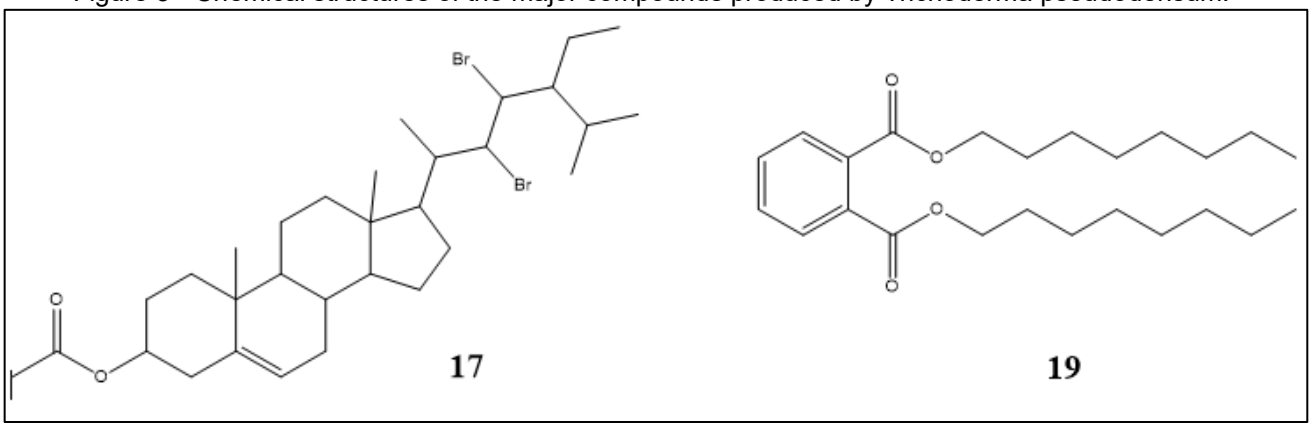
20	BIS(2-ayl-hexyl)benzeno-1,4-dicarboxylato	24,382	2,71	Ester
		Total	**100%**	

2 major compounds were identified, named as 22,23-dibromostigmasterol acetate (17), with retention time of 22.16 minutes and area of 11.26%. And di-n-octyl phthalate (19) (Figure 3), with a retention time of 22.67 minutes and an area of 44.46%. This present study is in agreement with that reported by Li et al. (2019), in which some compounds similar to those identified in the extract obtained from T. pseudodensum were isolated from strains of the genus Trichoderma, such as dibutyl phthalate isolated from strains of T. citrinoviride cf-27 (Li et al., 2019). And stigmasterol and palmitic acid isolated from T. harzianum T-4 and T. koningii T-8 (Li et al., 2019).

Figure 3 - Chemical structures of the major compounds produced by Trichoderma pseudodensum.



Source: Author, 2024.

Phthalic acid esters (PAEs), commonly referred to as phthalates recently, have attracted global attention due to their high production volume, widespread use in consumer products, and deleterious health effects. Widely used as a plasticizer, phthalates are not covalently bonded to the plastic matrix and can easily leach products into the surrounding environment. Public health concerns of these ubiquitous environmental contaminants include carcinogenic, teratogenic, hepatotoxic, and endocrine-disrupting properties. Some phthalates are already reported to be reproductive and developmentally toxic in animals and suspected endocrine-disrupting diseases in humans. Among others, di-(2-ethylhexyl) phthalate (DEHP) and di-n-octyl phthalate (Dn-OP) (19), have been listed as core pollutants by several regulatory agencies due to their low water solubility, high persistence in the environment, and toxicity to the liver, kidney, thyroid, and immune system (Sarkar et al., 2012).

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Stigmasterol and its class of compounds are known to possess various pharmacological activities. Stigmasterols are an active part of cyanobacterial extracts that exhibit antimicrobial and cytotoxic activities. Stigmasterols with the organic derivatives linked to the central protein, historically found to have antifungal properties (Karthik et al., 2015).

ALELOPATHIC ACTIVITY

The allelopathic assay was performed in triplicate using Petri dishes with four concentrations 0, 100, 500 and 1000 mg L-1, where 0 was designated as control (white). Lettuce seeds (Lactuca sativa L.) were incubated for a period of five days.

An important aspect to characterize an allelopathic activity is the influence of the extract on the length of the seedlings. Then, after five days of inoculation, the percentage of germinated seeds was evaluated and seedling lengths were measured, where it was evident that the crude extract demonstrated germination inhibition activity of lettuce seeds. Afterwards, the calculations of the means of root length and total length (Table 2) were performed using the Sisvar program using Tukey's test at 5% probability.

Table 2 – Mean values of root length (mm) and total length (mm) of lettuce seedlings treated with crude extract of *Trichoderma pseudodensum*.

	Average root length (mm)	Average Total Length (mm)
Treatment	Extract*	Extract*
Control	30.58 a3	43.80 a2
100 mg $^{L-1}$	19.08 a2 a3	27.85 a1 a2
500 mg $^{L-1}$	3.57 to 1 to 2	11.12 a1
1000 mg $^{L-1}$	1.92 a1	7.51 to 1
CV (%)**	47,19	40,62

*Means followed by the same letter in the columns do not differ from each other by Tukey's test at 5% significance. **CV = Coefficient of variation.

The means of each extract concentration were also submitted to linear regression analysis by the Sisvar program represented in Figure 4.

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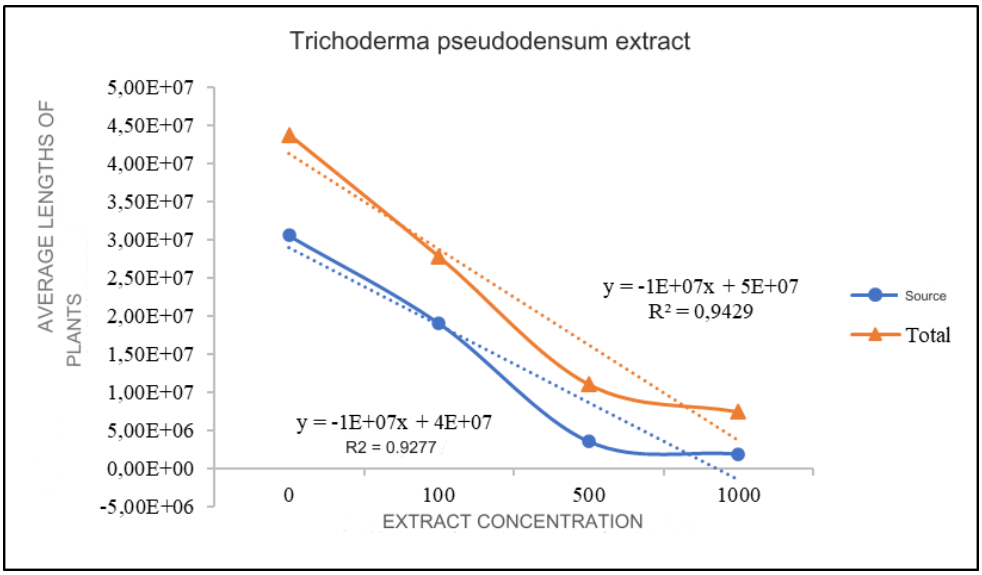
Figure 4 – Linear regression plot of the allelopathic activity of the crude extract of *Trichoderma pseudodensum*.



Source: Author, 2024

Trichoderma pseudodensum extract demonstrated significant inhibitory activity in the development of lettuce seedlings and roots. Inhibition began at the lowest concentration tested (100 mg L^{-1}) and progressively increased, reaching 82.8% inhibition in seedling development and 93.7% in roots at the highest concentration (1000 mg L^{-1}), compared to the control. The data analysis revealed a strong correlation between the concentration of the extract and the inhibitory effect, with coefficients of determination (R^2) of 0.93 for the length of the roots and 0.94 for the total size of the seedlings.

According to Fujii and Hiradate (2007), the allelopathic effects caused in plants occur through substances produced by secondary metabolites of the alkaloid, coumarin and phenolic compound classes, some of which have been identified in the extract of T. pseudodensum, and may be associated with the allelopathic effects observed.

ANTIFUNGAL ACTIVITY

A screening of the antifungal activity of the crude extract of Trichoderma pseudodensum in BDA medium was performed against the phytopathogens Sclerotinia sclerotiorum and Curvularia lunata, in triplicate. The inhibitory activity of the extract was observed by measuring the diameters of the colonies. With the data obtained, the percentage of mycelial growth inhibition (PIC) was calculated. The extract showed an inhibition of 74.53% against S. sclerotiorum and 14.72% against C. lunata, showing a greater antifungal activity against S. sclerotiorum (Figure 5).

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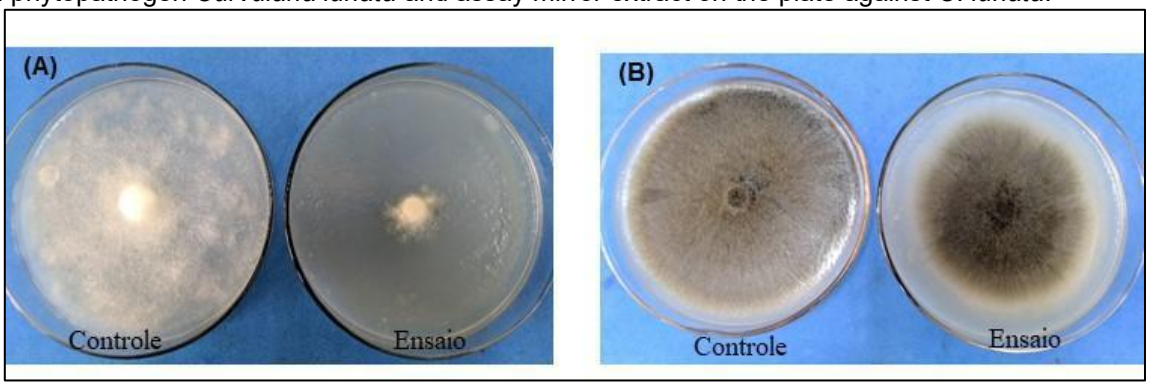
Figure 5 – Assay of the antifungal activity of *Trichoderma pseudodensum* extract in BDA medium. (A) Control *phytopathogen Sclerotinia sclerotiorum* and mirror extract assay on the plate against *S. sclerotiorum*. (B) control phytopathogen *Curvularia lunata* and assay mirror extract on the plate against *C. lunata*.



Source: Author, 2024.

Among the phytopathogens of great agronomic importance, the "white mold" (Sclerotinia sclerotiorum) stands out, which attacks the lower part of the soybean, corn, sorghum and vegetable plants, causing white lesions with accumulation of water in the affected vegetative organs. This fungus has sclerotium as a form of resistance, which can remain viable in the soil for up to 11 years. S. sclerotiorum attacks about 400 plant species in 270 genera belonging to 75 families, among these, a large part of which is used for agricultural purposes in the production of oil, feed, and fruits (Filho et al., 2020).

Strains of the genus Trichoderma, such as T. viride and T. harzianum, are used in the biological control of S. sclerotiorum (Guo et al., 2023; Yao et al., 2023). Generally, Trichoderma can invade the hyphae of S. sclerotiorum, adhere to and wrap around the hyphae, and rupture until they disintegrate (Yao et al., 2023). Compounds produced by Trichoderma strains showed antifungal activity against S. slerotiorum, such as harzianic acid, a tretramic acid produced by T. harzianum M10 (Li et al., 2019).

In addition, Akhtar, Javaid, and Qreshi (2020) evaluated the antifungal activity of extracts from the plant Sisymbrium irio against the phytopathogen Fusarium oxysporum f. sp. cepae, which causes the disease basal rot in onion cultivars. The leaf extracts showed antifungal potential due to the presence of bioactive compounds, such as γ-sisterol, and di-n-octyl phthalate, a compound identified as the major one produced by T. pseudodensum. This may explain the antifungal activity presented by the extract against S. slerotiorum.

CONCLUSION

The results of this study highlight the significant biotechnological potential of Trichoderma pseudodensum, especially in the biocontrol of phytopathogens and in the

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inhibition of plant growth through allelochemical compounds. The high inhibition of Sclerotinia sclerotiorum of 74.53% and the impact on the development of lettuce seedlings with inhibition of seedling development by 82.8% and root development by 93.7%, reinforce the ability of this fungus to produce bioactive compounds with promising applications in sustainable agriculture.

The chemical characterization revealed the presence of important metabolites, such as di-n-octyl phthalate, which has antifungal activity reported in the literature. This suggests that the crude extract of T. pseudodensum can be explored as an efficient tool both in pest control and in the modulation of plant growth, offering new opportunities for the development of more ecological agricultural products.

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