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Evaluation of the phytotoxic effect of extracts from endophytic fungi *Colletotrichum dianesei* and *Xylaria* sp. isolated from *Palicourea corymbifera* (Rubiaceae)

Evaluación del efecto fitotóxico de extractos de los hongos endofíticos *Colletotrichum dianesei* y *Xylaria* sp. aislados de *Palicourea corymbifera* (Rubiaceae)

Avaliação do efeito fitotóxico dos extratos dos fungos endofíticos *Colletotrichum dianesei* e *Xylaria* sp. isolados de *Palicourea corymbifera* (Rubiaceae)

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Abstract

The allelopathic action can be verified by the presence of phytotoxic substances, molecules that are directed to the agribusiness sector. The objective of this study was to evaluate the chemical composition of the methanolic extracts from *Colletotrichum dianesei* and *Xylaria* sp., and phytotoxic potential on *Lactuca sativa*. The fungi were grown in Sabouraud liquid medium supplemented with 0.2% yeast extract, under stirring. The mycelia were subjected to solvent extraction in order of increasing polarity, but methanol extracts were used in this investigation. The allelopathic activity was evaluated at a concentration of 1000 µg/mL in quadruplicate, evaluating: percentage and speed of germination, and growth of lettuce seedlings under laboratory conditions. *C. dianesei* extract reduced germination percentage by 36% and delayed germination speed by 63.8%, while *Xylaria* sp. didn't interfere with the germination percentage, it only delayed the germination rate by 43.9%. Root growth was negatively affected in 30.5% and 51.1% under the influence of extracts from *Xylaria* sp. and *C. dianesei*, there was also a reduction of 33.1 and 31.8% in the aerial part length, respectively. The thin layer chromatography showed the presence of aromatic substances and terpenes in both extracts, and alkaloids only in *C. dianesei* extract. The ^{1}H NMR spectra showed signals between 6 and 8.5 ppm (aromatic substances), being relatively more intense for *Xylaria* sp., signals between 3 and 4 ppm (sugars, showing anomers only for *Xylaria* sp.) and methyl groups between 0.7 and 1.3 ppm, suggesting terpenes. The results suggest that the fungal species evaluated have phytotoxic potential, and produce terpenes and aromatic substances.

Keywords: phytotoxicity, bioprospecting, endophytic

Resumen

La acción alelopática puede verificarse por la presencia de sustancias fitotóxicas, moléculas que están dirigidas al sector agroindustrial. El objetivo de este trabajo fue evaluar la composición química de los extractos metanólicos de *Colletotrichum dianesei* y *Xylaria* sp. y su potencial fitotóxico sobre *Lactuca sativa*. Los hongos se cultivaron en medio líquido Sabouraud suplementado con extracto de levadura al 0,2 %, en agitación. Los micelios se sometieron a extracción con disolventes en orden de polaridad creciente, pero en esta investigación se usaron los extractos metanólicos. La actividad alelopática de los mismos se evaluó a una concentración de 1000 µg/mL en cuadruplicado, evaluando: porcentaje y velocidad de germinación y crecimiento de plántulas de lechuga en condiciones de laboratorio. El extracto de *C. dianesei* redujo el porcentaje de la germinación en un 36 % y retrasó la velocidad de germinación en un 63,8 %, mientras que el extracto de *Xylaria* sp. no interfirió en el porcentaje de germinación, solamente retrasó en 43,9 % la velocidad de germinación. El crecimiento de las raíces se vio afectado negativamente en 30,5 % y 51,1 % bajo la influencia de los extractos de *Xylaria* sp. y *C. dianesei*; también hubo una reducción en la longitud de la parte aérea de 33,1 y 31,8 %, respectivamente. Las Cromatografías en capa fina mostraron la presencia de sustancias aromáticas y terpenos para ambos extractos, y alcaloides solo para el extracto de *C. dianesei*. Los espectros de RMN de ^{1}H mostraron señales entre 6 y 8,5 ppm (sustancias aromáticas), siendo relativamente más intensas para *Xylaria* sp., señales entre 3 y 4 ppm (azúcares, mostrando los anómericos solo para *Xylaria* sp.) y grupos metilas entre 0,7 y 1,3 ppm, sugiriendo terpenos. Los resultados sugieren que las especies de hongos evaluadas tienen potencial fitotóxico y producen terpenos y sustancias aromáticas.

Palabras clave: fitotoxicidad, bioprospección, endófitos

Resumo

A ação alelopática pode ser verificada pela presença de substâncias fitotóxicas, moléculas que são direcionadas ao setor agroindustrial. O objetivo deste trabalho foi avaliar a composição química dos extratos metanólicos de *Colletotrichum dianesei* e *Xylaria* sp. e seu potencial fitotóxico sobre *Lactuca sativa*. Os cogumelos foram

cultivados em meio líquido Sabouraud suplementado com 0,2% de extrato de levedura, sob agitação. Os micélios foram submetidos à extração por solvente em ordem crescente de polaridade, mas apenas os extratos metanólicos foram utilizados nesta investigação. A atividade alelopática foi avaliada na concentração de 1000 µg/mL em quadruplicado, avaliando-se: porcentagem e velocidade de germinação e crescimento de mudas de alface em condições de laboratório. O extrato de *C. dianesei* reduziu a porcentagem de germinação em 36% e atrasou a velocidade de germinação em 63,8%, enquanto a *Xylaria* sp. não interferiu na porcentagem de germinação, apenas atrasou a velocidade de germinação em 43,9%. O crescimento radicular foi negativamente afetado em 30,5% e 51,1% sob a influência dos extratos de *Xylaria* sp. e *C. dianesei*; também houve redução no comprimento da parte aérea de 33,1 e 31,8%, respectivamente. As cromatografias em camada delgada mostraram a presença de substâncias aromáticas e terpenos para ambos os extratos, e alcaloides apenas para o extrato de *C. dianesei*. Os espectros de RMN de ^{1}H mostraram sinais entre 6 e 8,5 ppm (substâncias aromáticas), sendo relativamente mais intensos para *Xylaria* sp., sinais entre 3 e 4 ppm (açúcares, mostrando os hidrogênios anoméricos apenas para *Xylaria* sp.) e grupos metila entre 0,7 e 1,3 ppm, sugerindo terpenos. Os resultados sugerem que as espécies fúngicas avaliadas possuem potencial fitotóxico e produzem terpenos e substâncias aromáticas.

Palavras-chave: fitotoxicidade, bioprospecção, endófitos

1. Introduction

Brazil plays an important role in the production and commercialization of food, especially in the cultivation of plant species that are relevant to the country's economy[1]. The biological control of unwanted species is one of the limiting factors for the establishment of crops, so the continuous and indiscriminate application of synthetic herbicides raises great concern in view of the negative consequences for agricultural ecosystems and possible contamination in food[2].

It is necessary to develop efficient and low-cost technologies capable of minimizing negative environmental impacts. In this sense, research using natural products has stood out, mainly exploring alternative sources in the production of allelochemicals[3].

Endophytic fungi are inserted in this scenario, and have received attention mainly for their competence in the production of secondary metabolites with wide applicability by the industry[4]. In the interaction between endophytic fungi and plant, biochemical and physiological machineries are adjusted in order to favor survival advantages for both[5][6]. Endophytes can increase the competitive capacity of hosts, especially through the production of phytotoxic substances[7].

For the preliminary screening of phytotoxic molecules, the germination rate and the root elongation in seeds are the established parameters[8][9]. The assay using *Lactuca sativa* is a reliable bioindicator, particularly because it is simple, inexpensive and requires a relatively small amount of sample[10].

In the search for bioactive molecules in endophytes, it is important to consider the chemical and biological history of the family, genus and species of the host plant; since fungi may have the capacity to produce secondary metabolites similar to those of their hosts[11].

The vegetable genus *Palicourea* is known for the occurrence of several substances exhibiting toxicity for animals, and phytotoxic properties[12][13][14]. The species *Palicourea corymbifera* has biotechnological potential confirmed either by the occurrence of secondary metabolites and by its medicinal and toxic properties[15][16]. However, studies aiming to evaluate the phytoxicity of substances produced by endophytic fungi associated to this plant have not yet been reported in the literature.

Thus, this work was carried out with the intent of exploring the biotechnological potential of two endophytic fungi isolated from *Palicourea corymbifera*, being: *Colletotrichum dianesei* and *Xylaria* sp., regarding the production of secondary metabolites; as well as evaluating the phytotoxic action of methanol

extracts on germination and growth of *Lactuca sativa*.

2. Materials and methods

2.1 Isolation and cultivation of fungi

The isolated endophytic fungi were obtained from leaves of the species *Palicourea corymbifera* (Rubiaceae) collected in September of 2015 in the Reserva Florestal Adolpho Ducke, Manaus, AM, Brazil (2°56'58.2"S 59°57'43.1"W). The samples were transferred to the Bioprospection and Biotechnology Laboratory located at INPA.

The leaves were subjected to pre-disinfestation, being washed with neutral soap under running water. Subsequently, the disinfestation stage began, carried out in a biological safety cabinet. The disinfestation steps were: alcohol 70% (1 minute), sodium hypochlorite 2.5% (4 minutes), alcohol 70% (1 minute), and washing 3 times in sterile distilled water. 500 µL of the last water was removed from the disinfestation wash and inoculated into three plates containing Agar Sabouraud (SB) culture media, in order to verify the efficiency of the method for the elimination of the epiphytic microbiota. After disinfestation, the leaves were cut into small fragments with the aid of a scalpel and tweezers. The obtained fragments were inoculated in Petri dishes containing SB culture medium plus oxytetracycline antibiotic (125 µg/mL), and then incubated in a BOD incubator at 30 °C for 48 hours.

After 48 hours of inoculation of the fragments, it was possible to begin the isolation of the emerged colonies. These were transferred to new SB culture media in order to purify the colonies. Afterwards, the isolates were preserved by the Castellani method and by cryopreservation.

Fungi were identified, by molecular methods, as *Xylaria* sp. and *Colletotrichum dianesei*. A similar genomic sequence for *Xylaria* at the species level in the Data Bank was not found, so it remained as sp. These were grown in Sabouraud Dextrose Broth medium with 0.2% yeast extract (SBL). For each fungus, 12 Erlenmeyer flasks of 500 mL containing 300 mL of culture medium were used. The vials were placed in a shaker incubator at 30 °C, with shaking at 120 rpm for 20 days.

No genomic sequence was found in the database at the species level within the genus Xylaria, therefore it remained as sp.

2.2 Extraction

After the growth period, extracts were obtained. First, the mycelia were separated from the growth media by filtration. Then, the mycelia were dried and mechanically milled in a pistil. To obtain the intracellular contents, the mycelia were placed in vials containing first dichloromethane (DCM), and extracted using an ultrasound bath for 20 min. The mycelia were then filtered and re-extracted with DCM by the same procedure two more times. Then mycelium was extracted with ethyl acetate (EtOAc) and finally with methanol (MeOH). All procedures repeated three times for each solvent.

The extracts of *Xylaria* sp. and *Colletotrichum dianesei* were concentrated in a rotary evaporator and subsequently dried in a fume hood. Only the methanolic extract was assayed, due to mass availability.

2.3 Germination bioassay

For the assay, methanolic extracts from *Xylaria* sp. and *Colletotrichum dianesei* were diluted in methanol at concentration of 1000 µg/mL. As substrate, filter paper discs impregnated with 2 mL of each extract were put into 9 cm Petri dishes. After all solvent evaporation, 2 mL of sterile water were added to humidify the substrate[17].

Each filter paper disc received 25 seeds (pre-sterilized, selected by uniformity in size and distributed evenly) of the target species *Lactuca sativa* (lettuce), with an average germination rate of 97%. Four plates with 25 seeds each were used, totaling 100 seeds per concentration (1000 µg/mL) of each tested extract[18].

In the control plates, an identical procedure was performed, replacing the 2 mL of extract with 2 mL of methanol, and after the solvent evaporation, adding 2 mL of sterile water.

The plates were placed into a germination room, where they stayed for an average of 10 days at a photoperiod of 16:8 hours (light/dark), at a

temperature of 26 ± 2 °C. The germination percentage assessment was carried out daily and the criterion used was the presence of visible protrusion. The experiment was concluded after three consecutive days without germination.

2.4 Growth bioassay

From the total of germinated seeds in each Petri dish, 10 seedlings were randomly selected. Three days after the root protrusion, measurements of the lengthening of the hypocotyl, coleoptile and radicle of each seedling were performed, using graph paper. 40 seedlings were analyzed per concentration (1000 µg/mL).

2.5 Data analysis

The following was evaluated: PG —percentage of germination (percentage of seeds germinated in each treatment)—, GSI —germination speed index (average number of seeds germinated per day in each treatment, expressed by the following formula: GSI = (G1 / N1) + (G2 / N2) + ... + (Gn / Nn), where: G1 = number of seeds germinated at the first count, N1 = number of days from the first count, G2 = number of germinated seeds in the second count, N2 = number of days elapsed until the second count, n = last count)—, and growth of aerial part (hypocotyl/coleoptile) and radicle[19].

The results obtained were analyzed using simple variance analysis (ANOVA), and the Tukey test was employed to compare means at 5% probability (p < 0,05). All analyzes were performed using the GraphPad Prism program[20].

2.6 Comparative thin layer chromatography and hydrogen nuclear magnetic resonance analysis

The methanolic extracts of *Xylaria* sp. and *Colletotrichum dianesei* were analyzed by TLC, using aluminum plates with silica gel with 254 UV indicator and dichloromethane/methanol 9:1 as eluent. The plates were revealed with UV lights (254 and 365 nm), iodine, anisaldehyde, ferric chloride, NP-PEG and Dragendorff.

All extracts were analyzed by ^{1}H Nuclear Magnetic Resonance (300 MHz, Fourier-300, Bruker).

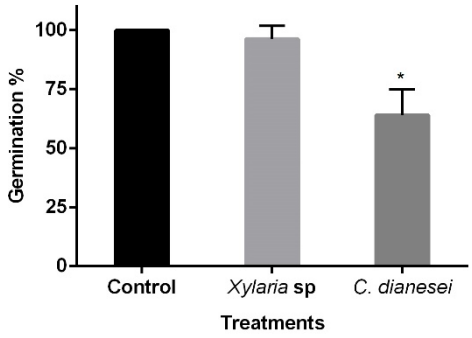
2.7 Transparency of data

Available data: The entire data set that supports the results of this study was published in the article itself.

3. Results

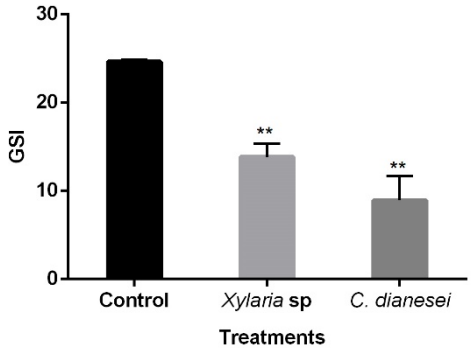
It was possible to verify a reduction of 36% on germination percentage when the seeds were submitted to the extract of *Colletotrichum dianesei* (Figure 1). The germination speed was delayed by 63.8% for *C. dianesei*, and by 43.9% for *Xylaria* sp (Figure 2).

Figure 1. Percentage of *Lactuca sativa* seed germination under the influence of the methanolic extract of *Xylaria* sp. and *C. dianesei*



Legend: * (p < 0.5).

Figure 2. Germination speed index (GSI) of *Lactuca sativa* seeds under the influence of the methanolic extract of *Xylaria* sp. and *C. dianesei*

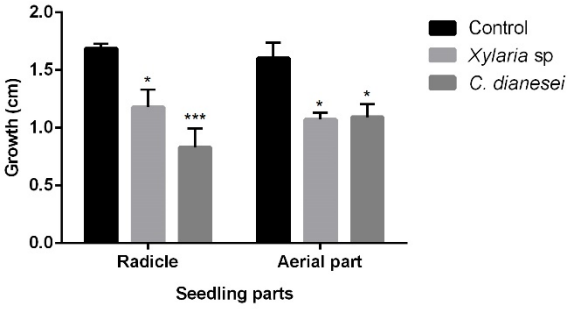


Legend: ** (p < 0.001).

Root and aerial part growth (hypocotyl + coleoptile) suffered negative growth interference by the

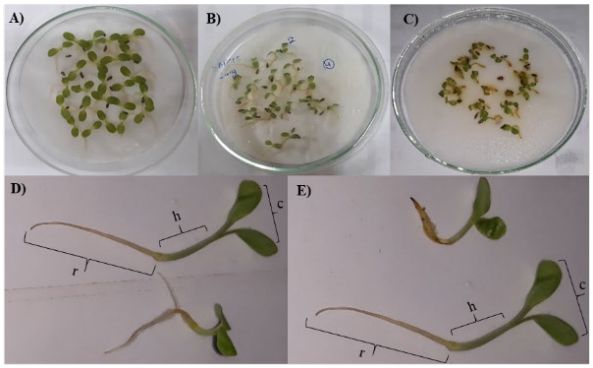
methanolic extracts of the two fungi tested (Figure 3). Growth interference can be qualitatively determined, and the presence of the beginning of necrosis in some *Lactuca sativa* seedlings submitted to the methanolic extract of the fungus *C. dianesei* is notorious (Figure 4).

Figure 3. Root and aerial part growth of *Lactuca sativa* with *Colletotrichum dianesei* and *Xylaria* sp. methanolic extracts



Legend: * ($p < 0.5$), *** ($p < 0.0001$).

Figure 4. Interference of *Colletotrichum dianesei* and *Xylaria* sp. methanolic extracts on the growth of *Lactuca sativa*



Legend: A) Control plate. B) Seeds submitted to the methanolic extract of *Xylaria* sp. C) Seeds submitted to the methanolic extract of *Colletotrichum dianesei*. D) Seedlings comparison between control and fungus *Xylaria* sp. E) Seedlings comparison between control and with *C. dianesei* fungus. The letters on the control seedlings correspond to: r: radicle, h: hypocotyl, and c: coleoptile.

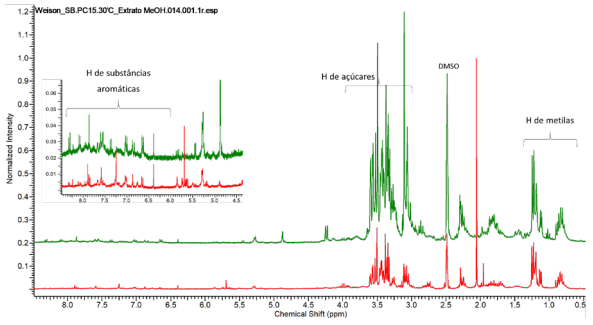
TLC analysis indicates the presence of aromatic substances and alkaloids (only for *C. dianesei*). Terpenes were on both fungi methanolic extracts, but higher amounts on *Xylaria* sp. (Table 1).

Table 1. TLC comparison of *Colletotrichum dianesei* and *Xylaria* sp. methanolic extracts

Fungus / Revelator	*C. dianesei*	*Xylaria* sp.
UV light 254 nm	Present (Less intense)	Present (Plus intense)
UV light 365 nm	Present (Few spots)	Present (Few spots)
Draggendorff (alkaloids)	Present	Absent
Ferric chloride (aromatic substances)	Present	Absent
Anisaldehyde (general/terpenes)	Present (Less spots)	Present (Plus spots)
NP-PEG (flavonoids)	Absent	Absent

Hydrogen nuclear magnetic resonance (^{1}H-NMR) analysis confirmed the TLC analysis, since there are signs in the region of 6 and 8.5 ppm (aromatic substances), being relatively more intense for *Xylaria* sp., signs between 3 and 4 ppm (sugars, showing anomeric hydrogen only for *Xylaria* sp.) and signs between 0.7 and 1.3 ppm, suggesting methyl groups of terpenes (Figure 5).

Figure 5. ^{1}H NMR analysis of *Colletotrichum dianesei* and *Xylaria* sp. methanolic extracts



Legend: Xylaria sp., and Colletotrichum dianesei

4. Discussion

Regarding the parameters evaluated in allelopathic activity, germination has shown to be less sensitive to allelochemicals, with greater interferences being evidenced in the speed of germination and in the growth of several species[21]. This corroborates with the test performed in this study, in which there was no interference in the germination of the seeds submitted to *Xylaria* sp., but there was a negative influence on the germination speed and growth.

Other phytotoxic evaluations have already been carried out with species of the genus *Xylaria* and *Colletotrichum*. The AcOEt extract from the culture filtrate, without cells, of *Colletotrichum dematium* showed phytotoxic action against *Parthenium hysterophorus* L.[22]. And the substances (3aS, 6aR) - 4,5-dimethyl-3,3a,6,6a-tetrahydro-2Hcyclopenta[b] furan-2-one and myrothe-ciumone A, isolated from the ethyl acetate extract of *Xylaria curta* broth, presented inhibitory effects against *Lactuca sativa*, both of growth and germination, in concentrations below 200 µg/mL, these substances being considered phytotoxic[23].

A reduction in the growth of test plants was also noted by Spiassi and collaborators[24], where the fungus *Fusarium graminearum*, *Macrophomina phaseolina* and *Diplodia maydis* promoted a reduction on root and aerial parts growth of "amendoim bravo" (*Euphorbia heterophylla*).

Considering a 50% inhibition or stimulus as a satisfactory standard in the evaluation of phytotoxic activities, interesting results were evidenced for the methanolic extract of *C. dianesei*, in which the germination speed of *L. sativa* was reduced by 63.8%; and root growth inhibited by 51.1% in lettuce seedlings.

Among some of the chemical classes with great interference in plant development processes are phenolic substances, which act on enzymes that coordinate various physiological processes, and terpenes, which inhibit germination and plant growth[25].

5. Conclusions

It can be concluded that the fungus *Colletotrichum dianesei* interfered negatively against the tested target plant. Chemical analysis showed evidences of terpenes and aromatic substances, chemical classes known by their allelopathic action.

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Author contribution statement

All authors contributed equally to the content.

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