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Antifungal Activity of Extracts of *Cladosporium cladosporioides* (Fres) de Vries

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The influence of microorganisms on the quality of coffee is usually studied using the fungalgenus*Cladosporium*. This fungus is offundamental importance to coffee cultivationbecause it is associated with good quality drinks. This research was carried out to determine the effect of extracts of *C. cladosporioides* on a BDBionutrients™ medium extracted with extractors (methanol, ethanol, dimethyl sulfoxide and ethyl acetate) and the effects of their activities against fungi on coffee quality. Sporulation and germination tests were performed with extracts, and their effects against the fungi *Aspergillusniger*, *Aspergillusochraceus, Fusarium sp.* and *Penicillium sp*. were evaluated for changes in the morphology of fungi, such as changes in colour, size, spore production and inhibition of germination.This experiment was carried out in a completely randomized design, with six replicates and ten replicates for testing sporulation and germination respectively. Tests have demonstrated the potential of the metabolites obtained for sporulation and germination of *A.ochraceus* from *C.cladosporioides* metabolites extracted withmethanol, activity of 83% and31% respectively; for *A.niger*metabolites extracted with ethyl acetate, activity of 54% and 60%; for*Fusarium sp.* metabolites extracted with methanol, activity of 87% and 40%; and for the fungus*Penicillium sp.*,activity of 79% and 19% respectively.

Key words: Fungi, metabolites, extractor, sporulation, germination.

Currently, natural product chemists are finding new sources of wide biological diversity. Fungi are organisms of enormous diversity of species that inhabit the various existing plants and fall within the search for new compounds with chemical and biological variability. Thus, there is great potential for chemical and biological studies to be explored with the secondary metabolites produced by fungus[1] and bacteriawith antimicrobial activity against some pathogenic microorganisms[2].

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Several of the microorganisms used as biological control agents produce secondary metabolites that affect growth and germination through the production of antibiotics and/or enzymes that degrade cell walls. According to several reports, many fungi species are capable of causing antibiosis to other pathogenic organisms through the synthesis and excretion of toxic metabolites[3]. Among the composts produced by fungi, cladosporol, a substance synthesized primarily by *Cladosporium* species, has been studied.

In this context, work related to the investigation of multiple mechanisms of biocontrol of *Cladosporium sp*. establishes the chemical characterization of secondary metabolites produced by strains of the fungus and its activity by inhibiting the urediniospore sporulation of the

rust agent *Uromyces appendiculatus*[4]. Assante *et al.*[5] found that *Cladosporium tenuissimum*, producer of cladosporol ($C_{20}H_{16}O_6$), known as an inhibitor of glucan biosynthesis, at concentrations of 12.5 and 100 ppm inhibited germination and mycelial growth of several fungi. Sakagami *et al.*[6] isolated Cladosporol (1) from the culture filtrate of *Cladosporium cladosporioides*. Cladosporol demonstrated inhibitor activity of biosynthesis of -1,3 glucans, and the structure of cladosporol was determined by spectroscopic analysis. The identification of new substances and biological activities already established with the cultivation of fungi indicate that these microorganisms are a potential source of bioactive substances and new, unexplored chemical entities.

Thus, our aim was to evaluate the antimicrobial activity of the fungus *Cladosporium cladosporioides* extract through sporulation and germination tests with *Aspergillus niger, Aspergillus ochraceus, Fusarium sp.* and *Penicillium sp.*

MATERIALS AND METHODS

General Procedures

Preparation and inoculation of fungi

The fungi *Aspergillus ochraceus, Aspergillus niger, Fusarium sp., Penicillium sp.* and *Cladosporium cladosporioides* were cultured in a potato dextrose agar (PDA) medium. All isolates were obtained from coffee beans benefiting from being deposited in the mycology collection of EcoCentroEpamig, Lavras, MG. After the transplant, the fungi were maintained in a germination chamber.

Cultivation of the microorganism

The cultures were prepared from the strain of *Cladosporium cladosporioides* in a PDA medium. After growth in an incubator at 30°C for approximately 10 days, the culture was refrigerated at 4°C and peaked every 30 days.

Preparation of extract

We obtained the fungal extract from the mycelium of *Cladosporium cladosporioides*. Mycelial discs (0.5 cm diameter) were removed from the culture medium containing BDA mycelial growth of *C.cladosporioides* and immersed in liquid potato dextrose (PD). After approximately 20 days of cultivation, under orbital agitation at 300 rpm at room temperature (28 to 30°C), the content of the flasks was filtered through filter paper GF/A. The medium was separated from mycelia by vacuum filtration using a Buchner funnel (Whatman Grade No. 1 filter paper), followed by centrifugation at 15 g for 15 minutes. The supernatant was sterilized by filtration through a 0.45 mM filter (Millipore Corp., Bedford, MA), followed by Millipore-type membrane filtration (0.2 mm in diameter of the pore), to obtain the filtered mycelial growth. The filtrate was subjected to partition with the extractors. This extraction involves obtaining organic material free of its original matrix (cells and tissues). The extraction of metabolites was obtained from efficient extractors, such as ethyl acetate (EtOAc), ethanol (Et(OH)), dimethyl sulfoxide (DMSO) and methanol (Me(OH)) (aliquots of 30 mL/L) immersed for 7 days (for the death of the fungus and extraction of metabolites).

Test in vitro sporulation of fungi

Based on the test in vitro sporulation of fungi, *A. ochraceus, A. niger, Fusarium sp.* and *Penicillium sp.*, with BDC, was added to 10 mL of sterile distilled water, forming a spore suspension. The suspension was added to the culture medium cast BDA by mixing and pouring them. Holes in the centre of the culture medium were utilised for the addition of filtrates and extracts. The plates were dried in the absence of germicidal light.

To test sporulation, 50 mL of extract + BDC were then added to each puller in the centre of Petri dishes with PDA medium + yeast suspension of *A. ochraceus, A. niger, Fusarium* and *Penicillium* and witnesses Et(OH), EtOAc, Me(OH) and DMSO. The plates were sealed and incubated in a germination chamber with a photoperiod of 12 hours. The plates were evaluated after 7 days of incubation. For the evaluation of tests, we added 40 mL of sterilized water to plate-incubated fractions containing BDC + fungus, which were then scraped to obtain a suspension. We carried out spore counts with a Neubawer chamber, fluorescent microscopy. We evaluated the morphological structures of fungi, such as changes in colour, size and spore production.

Test in vitro germination of spores

The germination test was prepared in Petri dishes of 6 cm containing 10 mL of medium 2% agar-water. The treatments were mixed in half and transferred about 50 microliters of the spore

suspension of each fungus. The plates were kept in a chamber at 250°C for 6 hours under light conditions, followed by 6 hours of dark. After the incubation period, lactoglicerol solution was used to analyse the germination of spores. A count was made at a depth where they were divided into quadrants with 50 to 100 spores per quadrant.

Evaluations were performed after 12 hours of incubation under a light microscope for the fungus *Aspergillus ochraceus*, after 18 hours for *Aspergillus niger*, after 24 hours to *Fusarium sp*. and after 26 hours for *Penicillium sp*.; evaluations were considered germinated spores if germination tubes were longer than the spore.

Experimental design and statistical analysis

The experiments with the fungi studied (*A. ochraceus, A. niger, Fusarium sp., Penicillium sp*.) were installed in a completely randomized design, with 9 treatments overall, 6 repetitions for sporulation tests and 10 repetitions for germination tests.

The data were subjected to analysis of variance using the software Sisvar [7]. The treatment means were compared by the Scott-Knott test at 5% probability.

Action of secondary metabolites of the filtrate of *C. cladosporioides* obtained from

Table 1. Effect of metabolites of the fungus *C. cladosporioides* on the germination of spores of the fungus *A.ochraceus*

Treatment Means (%)	
Treatments	Means (%)
Extractor + ExtractorAcOEt	19 a
Extractor + Extractor DMSO	20 a
Extractor + ExtractorEt(OH)	36 b
Extractor DMSO	40 b
Extractor + Extractor Me(OH)	44 b
Extractor Me(OH)	74 c
ExtractorAcOEt	74 c
ExtractorEt(OH)	82 c
TestemunhaGeral	96 d

CV: 22.27 * Means followed by same letter do not differ by Scott-Knott test at 5% probability

Table 2. Effect of metabolites of the fungus *C. cladosporioides* on the germination of spores of the fungus *A.niger*

Treatment Means (%)	
Treatments	Means (%)
Extractor + ExtractorAcOEt	3 a
Extractor + ExtractorEt(OH)	4 a
Extractor + Extractor Me(OH)	6,2 a
Extractor + Extractor DMSO	6,4 a
ExtractorEt(OH)	13 b
Extractor DMSO	28 c
Extractor Me(OH)	38 d
ExtractorAcOEt	40 d
TestemunhaGeral	62 e

CV: 24.54 * Means followed by same letter do not differ byScott-Knott test at 5% probability.

Table 3. Effect of metabolites of the fungus *C. cladosporioides* on the germination of spores of the fungus *Fusariumsp*

Treatment Means (%)	
Treatments	Means (%)
Extractor + ExtractorEt(OH)	40 a
ExtractorEt(OH)	49 b
Extractor + Extractor Me(OH)	58 c
Extractor + Extractor DMSO	70 d
Extractor + ExtractorAcOEt	72 d
Extractor DMSO	88 e
ExtractorAcOEt	91 e
Extractor Me(OH)	96 e
TestemunhaGeral	96 e

CV: 11.81 * Means followed by same letter do not differ by Scott-Knott test at 5% probability.

Table 4. Effect of metabolites of the fungus *C. cladosporioides* on the germination of spores of the fungus *Penicilliums*p

Medium treatment (%)	
Treatments	Means (%)
Extractor + ExtractorEt(OH)	18 a
ExtractorEt(OH)	33 b
Extractor + ExtractorAcOEt	34 b
Extractor + Extractor Me(OH)	35 b
Extractor + Extractor DMSO	45 c
ExtractorAcOEt	45 c
Extractor Me(OH)	51 c
Extractor DMSO	60 d
TestemunhaGeral	84 e

CV: 25.72 *Means followed by same letter do not differ by Scott-Knott test at 5% probability.

extraction with Me(OH), Et(OH), DMSO and EtOAc on sporulation of the fungi *Aspergillus ochraceus, Aspergillus niger, Fusarium sp.* and *Penicillium sp.*

According to the results expressed in Figure 1, the influence of solvents on the extraction of metabolites from culture filtrates of *C.cladosporioides* can be observed after microscopic and statistical analysis.

Sporulation of *A. ochraceus* with witnesses (DMSO, Et(OH), Me(OH) and EtOAc), in the absence of the extract, compared with the control, generally showed different levels of reduction on sporulation. In the presence of the extract BDC + extractors, one can analyse the action of metabolites extracted from the filtrate, and their activity in reducing sporulation of the fungus, as higher than the extractants and the control.

The extracts showed significantly lower levels of sporulation to the witness. However, the extractors also had different levels of inhibition of sporulation.

As the extractor Me(OH) presented the lowest reduction of sporulation when compared with control groups, we can deduce that the greater inhibition of the extract was due to the action of the metabolite of the fungus.

While the extractor DMSO showed lower extraction capacity of the metabolite compared with other solvents to the fungus *Aspergillus ochraceus*, the extractor DMSO has low toxicity and strong penetration capacity, and many substances, when combined with DMSO, can be carried through the membranes. That final characteristic is the result of its ability to interact or combine with nucleic acids, carbohydrates, lipids, proteins and many drugs, so it induces no irreversible change in molecular configuration. With respect to in vitro assays, it can be said that the DMSO only served as vehicle in vitro tests with fungi, as observed by Souza *et al.*[8], Ferreira *et al*.[9] and Jacques *et al*.[10], according to a previous study of optimal concentrations for its use.

From Figure 2, considering that the extractor EtOAc showed the lowest inhibition of sporulation of *Aspergillus niger* in relation to the witness, it was found that the significant reduction of sporulation by the extract obtained with the same solvent showed the greater inhibitory action exerted by the extracted metabolite of the fungus *Cladosporium cladosporioides*. In contrast, extractors Et(OH) and DMSO for the fungus *Aspergillus niger* did not show good activity in the case of extraction of metabolites.

A comparison of the witnesses Et(OH) and DMSO and all witnesses reveals that the solvents themselves significantly inhibited sporulation of the fungus *A. niger*. Therefore, inhibition of sporulation of *A. niger* for the production of metabolites synthesized from the extraction with ethanol and DMSO was not considered efficient.

According to the results expressed in Figure 3, all the extracts significantly reduced sporulation of the fungus *Fusarium sp*. However, when analysing the results for the extractors used in isolation, it was found that they exerted a different action on sporulation of the fungus. Extractors Et(OH) and Me(OH), which presented low inhibitory activity on sporulation, can be assigned a highly significant inhibition observed in extracts obtained from solvent extracted for metabolites of the fungus. The solvent DMSO showed considerable inhibition of sporulation of *Fusarium sp.* when it acted in the absence of the extract of *C. cladosporioides*. Inhibition of sporulation of the fungus for the production of metabolites was synthetized from the extraction with DMSO, so it was not considered efficient.

It should be noted that, according to Figure 4, the extracts obtained from different solvents and extractants showed significantly higher rates of inhibition of sporulation of the fungus *Penicillium sp*. However, considering that extractors, when used alone, showed different degrees of inhibition of sporulation, it appears that the extractor Et(OH) presented the lowest rate of inhibition of sporulation and extractor Me(OH) did not differ from the control, indicating a failure to exercise a depressing effect on sporulation.

The results indicate that the inhibitory action exerted by the extracts obtained from these solvents was due, predominantly in the case of the first and only in the case of the second, to the metabolites of the fungus *Cladosporium cladosporioides* present in these extracts. Figure 5 shows the performance of secondary metabolites extracted from each extractor's effect on inhibition of sporulation of the fungus through the changes of macroscopic characteristics.

Action of the secondary metabolites of filtered *C. cladosporioides* obtained from extraction with Me(OH), Et(OH), EtOAc and DMSO were tested on the germination of *Aspergillus niger*. The results, as illustrated in Table 1, for the germination tests with the fungus *A. ochraceus*, from the germination efficiency of the extract, we can analyse that EtOAc presented as the best extractor due to its lower percentage (19%) in the

Fig. 1. Effect of metabolites extracted from fungus *C. cladosporioides* spores on the sporulation of the fungus *Aspergillus ochraceus*.(E/Extractor);(E + E/Extractor); (Extract *C.cladosporioides* + Extractor);Witness (without extraction process)

Fig. 2. Effect of metabolites extracted from the fungus *C. cladosporioides* spores on the sporulation of the fungus *Aspergillus niger*.(E/Extractor);(E + E/Extractor); (Extract *C.cladosporioides*+Extractor); Witness (without extraction process)

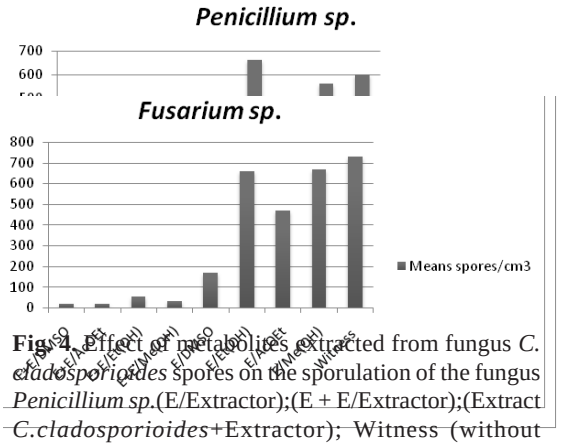

Fig. 3. Effect of metabolites extracted from the fungus *C. cladosporioides* spores on the sporulation of the fungus *Fusarium sp.*(E/Extractor);(E + E/Extractor); (Extract *C.cladosporioides*+Extractor); Witness (without extraction process)

Fig. 4. Effect of metabolites extracted from fungus *C. cladosporioides* spores on the sporulation of the fungus *Penicillium sp.*(E/Extractor);(E + E/Extractor);(Extract *C.cladosporioides*+Extractor); Witness (without extraction process)

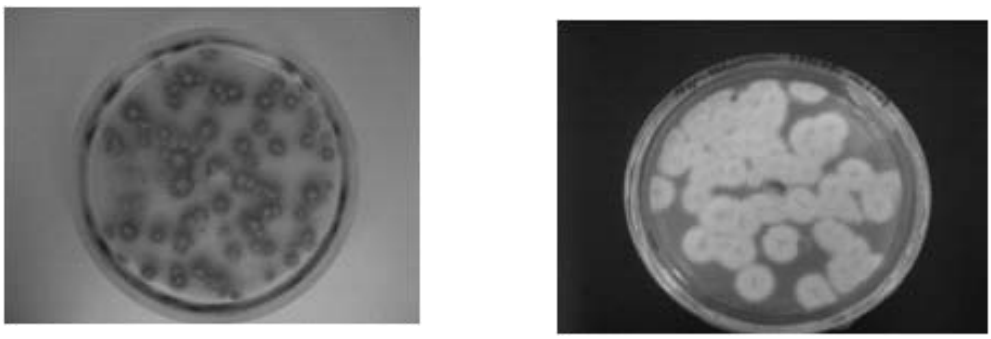

Fig. 5. Action of metabolites of *C. cladosporioides* on sporulation of the fungus *A. ochraceus*. a) Witness without extraction process and b) fungus extract obtained with Me(OH)

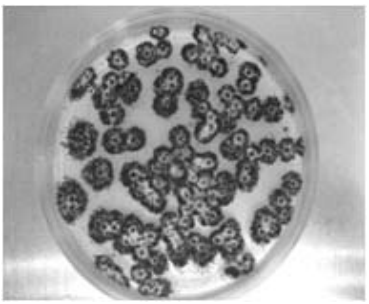

Fig. 6. Action of metabolites of *C. cladosporioides*on sporulation of the fungus *A. niger.* a) Witness without extraction process and b) fungus extract obtained with ethyl acetate.

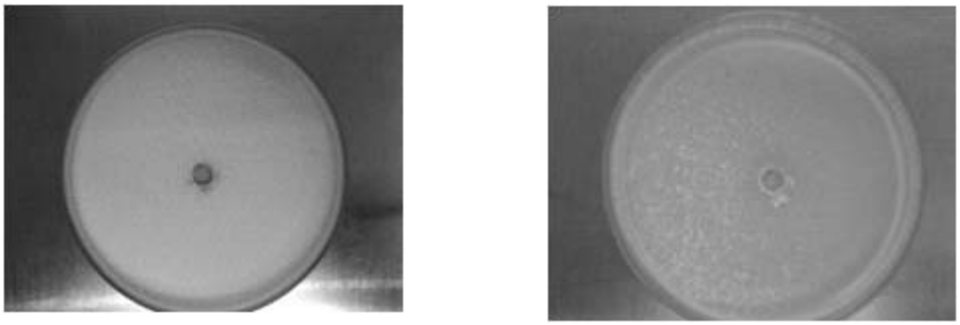

Fig. 7. Action of metabolites of *C. cladosporioides* on sporulation of the fungus *Fusarium sp*. a) Witness without extraction process and b) fungus extract obtained with methanol

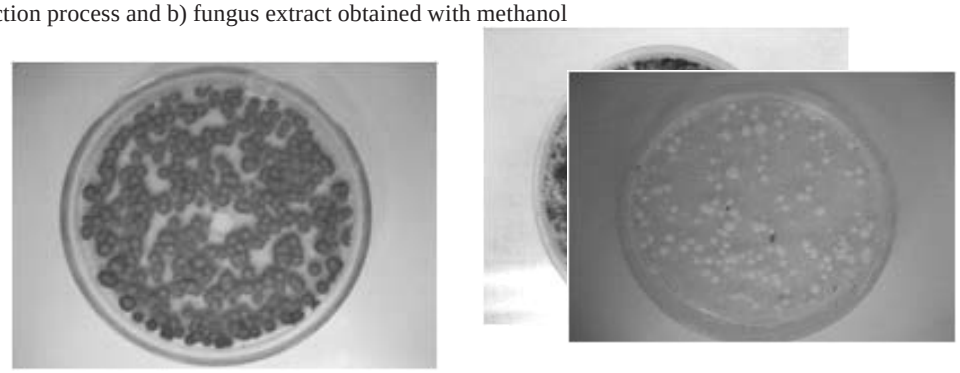

Fig. 8. Action of metabolites of *C. cladosporioides* on sporulation of the fungus *Penicillium sp*. a) Witness without extraction process and b) fungus extract obtained with Me(OH)

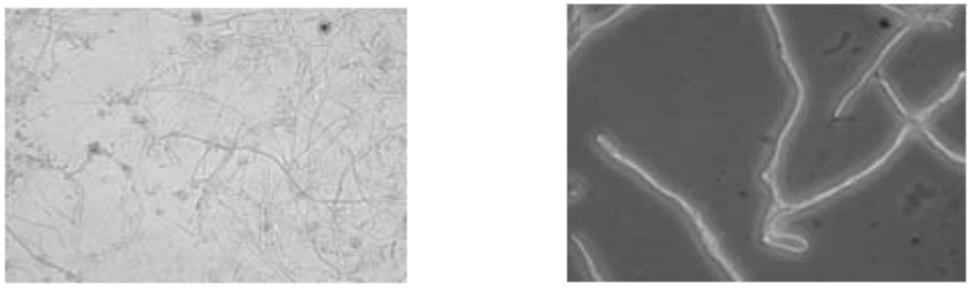

Fig. 9. Microphotograph of the optical microscope test germination of the fungus as *A.ochraceus*) a) without extraction process b) in the presence of extract *C. cladosporioides* extracted with EtOAc

germination of the fungus *A.ochraceus*.

The extract obtained with DMSO showed that although it behaved similarly to the extract obtained with the EtOAc extractor, the extractor when applied alone had a significant inhibitory effect on *A.ochraceus* germination. The extracts obtained with the extractants Et(OH) and Me(OH) exerted an intermediate effect and higher than the extractants used in isolation but which, in turn, had a significant depressive effect on spore germination when compared with the control. In the following illustration, it can be visualized in the optical microscope micrograph of the germination of the fungus *A. ochraceus.* In Figure 9, we can see a lot of germination spores, which are visible under the microscope several germ tubes. In Figure 9b, one can see the effect of the extract *C. cladosporioides'* inhibition of germination, which is revealed in the presence of only a few spores and germ tubes that did not germinate.

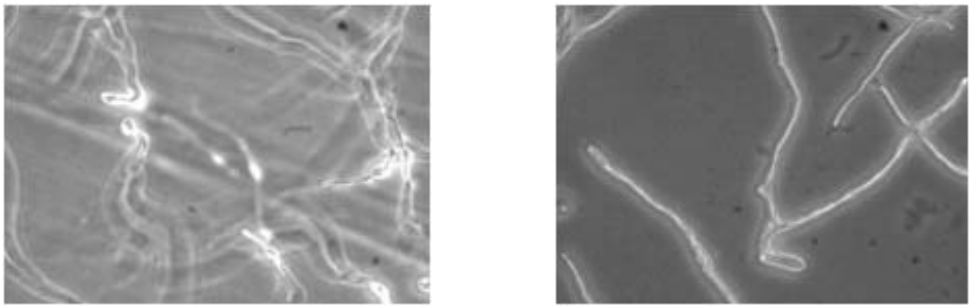

Fig. 10. Microphotographs of the germination of the fungus *A. niger*. a) Witnessed general without extraction process b) In the presence of metabolites of *C. cladosporioides* extracted with EtOAc.

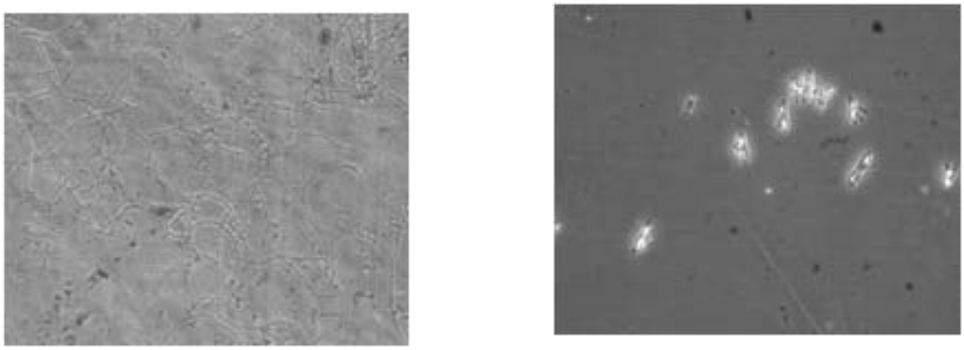

Fig. 11. Microphotograph of the optical microscope test germination of the fungus *Fusarium sp*. a) general witness b) in the presence of metabolites of *C. cladosporioides* extracted with methanol

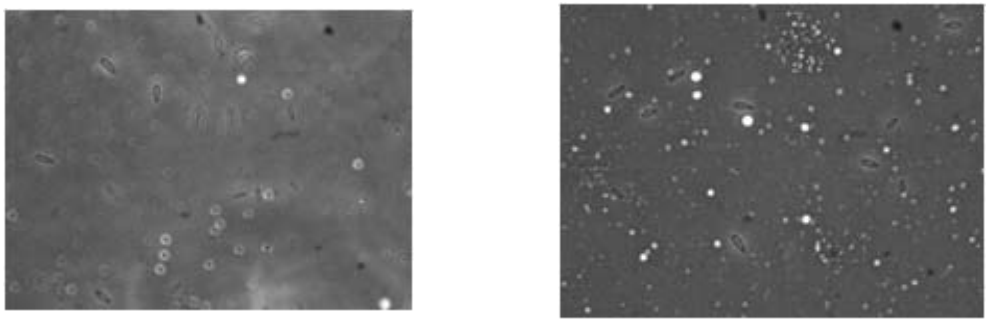

Fig. 12. Microphotograph of the optical microscope test germination of the fungus *Fusarium sp*. a) general witness b) in the presence of extract *C. cladosporioides* extracted with methanol

Action of the secondary metabolites of filtered *C. cladosporioides* obtained from extraction with Me(OH), Et(OH), EtOAc and DMSO on the germination of *Aspergillus niger*.

For the fungus *A.niger*, table 2, extracts from all extractants tested showed a significant reduction in spore germination compared to the control. However, when tested alone, extractors showed different intensities of inhibitory effects on germination. This effect was more pronounced in the case of extractors Et(OH) and DMSO, so we must assign the better effect to the metabolites extracted with solvents EtOAc and Me(OH).

The high rate of inhibition of germination of *A.niger* is achieved by the production of metabolites from the solvent extraction. The synthesis of secondary metabolites is associated with cell differentiation or development, as observed in yeast and filamentous growth that present with complex morphology.

In *A. niger*, the conidial cell wall is composed of two layers: an external hydrophobic layer containing melanin and rodlet proteins, and an internal layer, electron translucent, containing polysaccharides such as a- and b-glucans, chitin/chitosan and galactomannan[11]. Scanning electron microscopy and atomic force microscopy have demonstrated that the outer rodlet/melanin layer of the resting conidia is progressively lost, and the cell surface layer is changed into a layer of amorphous material, unmasking b1,3glucan on the surface of the conidium[12, 13, 14].

Figure 10 shows microphotographs of the germination of the fungus *A. niger*, taken through a light microscope. You can see in the microphotograph witness a greater number of germinating spores compared to the microphotograph in which the spores in the presence of metabolites extracted from ethyl acetate show fewer germ tubes and the presence of spores not germinated.

Action of the secondary metabolites of filtered *C. cladosporioides* obtained from extraction with Me(OH), Et(OH), EtOAc and DMSO were tested on the germination of *Fusarium sp*. The action of the fungal metabolite extracted by extractants is demonstrated to be lower for the fungus *Fusarium*, since the germination percentage was higher in all treatments as shown in Table 3.

The action of the fungal metabolite extracted by extractants was demonstrated to be lower for the fungus *Fusarium*, with a high germination percentage in all treatments. The whole extract + extractor Et(OH), although it revealed a 40% germination, showed a relatively minor rate compared with the germination rate of the witness generally. The active principles derived from secondary metabolites present in extracts of *C. cladosporioides* extracted with Et(OH) a share of only 9.3%, proving to be effective in this solvent.

According to Table 3, the metabolite extracted with Me(OH) reduced the germination rate from 96% to 58% compared to control groups, indicating a rate of action of the metabolite of 40%, a high value when analysing the values of action for each of the extractors. In the illustration below, non-germinated spores of *Fusarium sp.* can be seen in the presence of extract *C. cladosporioides* extracted with Me(OH).

Action of the secondary metabolites of filtered *C. cladosporioides* obtained from extraction with Me(OH), Et(OH), EtOAc and DMSO on the germination of *Penicillium sp*.

In the germination of the fungus *Penicillium sp*., as shown in Table 4, according to statistical analysis, the extractors had some interference in germination compared to the control overall. This indicates that the solvents without the action of the metabolite were responsible for reducing germination. However, when analysing the effect of these solvents, *C. cladosporioides* added to the extracts reduced the germination percentage to significantly lower than in the case of extracts obtained from the EtOAc solvent and Me(OH).

The other extracts displayed a significant reduction in the rates of germination compared to control, but with significant involvement in the process of extracting the inhibition of germination of the fungus.

The extractor Et(OH), as illustrated in Figure 12, significantly reduced the germination of the fungus, since germination in the absence of extractors was 84% and with interference extractor Me(OH) was increased to 33%. In the extract, the germination percentage was only 18%.

CONCLUSIONS

The extracts of the fungus *Cladosporium cladosporioides* (Fres) de Vries, obtained from the

solvents (EtOAc, Et(OH), Me(OH) and DMSO), reduced sporulation and germination of *Aspergillus ochraceus*, *Aspergillus niger*, *Fusarium sp*. and *Penicillium sp*. The metabolites showed a marked sporicidal action and significant reduction in spore germination, limiting the development of the fungi tested. The results obtained allow the conclusion that biological fungicides can be formulated through the combination of metabolites obtained from the different extractors used.

According to analysis to identify potential compounds of fungal extracts, a new stage of purification is performed to determine the possible fungal metabolites.

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