Antifungal activity of extracts from brazilian Cerrado plants on *Colletotrichum gloeosporioides* and *Corynespora cassiicola*

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ABSTRACT: This study aimed to determine the antifungal activity of leaf aqueous and hydroethanolic extracts of 10 plants from the Brazilian Cerrado on *Colletotrichum gloeosporioides* and *Corynespora cassiicola.* Antifungal activity was measured through the incorporation of each extract in a culture media or spore suspension, at 50% concentration relative to the volume, determining respectively the mycelial growth and the spore germination. Then, the percentages of mycelial growth inhibition and spore germination inhibition were obtained based on the comparison with the control. The extracts had a variable action on the phytopathogens, from mycelial growth stimulation for *Aristolochia esperanzae* and *Byrsonima verbascifolia* extracts to complete inhibition of mycelial growth and spore germination for *Myracrodruon urundeuva* and *Lafoensia pacari* extracts. *M. urundeuva*, *L. pacari* and *Caryocar brasiliense* leaf extracts had antifungal activity against *Colletotrichum gloeosporioides* and *Corynespora cassiicola*; the hydroethanolic extracts presented more antifungal activity than the aqueous extracts, and spore germination of both phytopathogens was more affected than their mycelial growth.

Key words: aqueous extract, hydroethanolic extract, spore germination, mycelial growth, alternative control

RESUMO: Atividade antifúngica de extratos de plantas do Cerrado brasileiro sobre *Colletotrichum gloeosporioides* e *Corynespora cassiicola*. O objetivo deste trabalho foi determinar a atividade antifúngica de extratos aquosos e extratos hidroetanólicos de folhas de 10 plantas do Cerrado brasileiro sobre *Colletotrichum gloeosporioides* e *Corynespora cassiicola*. A determinação da atividade antifúngica foi realizada pela incorporação do extrato em meio de cultura ou na suspensão de esporos, na concentração de 50% em relação ao volume, determinando-se, respectivamente, o crescimento micelial e a germinação de esporos. Em seguida, pela comparação com a testemunha, foram obtidas as percentagens de inibição do crescimento micelial e da germinação dos esporos. Foi constatado comportamento variável dos extratos sobre os fitopatógenos, desde o estímulo no crescimento micelial para os extratos de *Aristolochia esperanzae* e *Byrsonima verbascifolia*, até a inibição completa do crescimento micelial e dagerminação dos esporos para os extratos de *Myracrodruon urundeuva* e *Lafoensia pacari*. Extratos de folhas de *L. pacari*, de *M. urundeuva* e de *Caryocar brasiliense* apresentaram atividade antifúngica sobre *Colletotrichum gloeosporioides* e *Corynespora cassiicola*; os extratos hidroetanólicos proporcionaram mais atividade antifúngica que os extratos aquosos, e a germinação de esporos de ambos os fitopatógenos foi mais afetada que o crescimento micelial.

Palavras-chave: extrato aquoso, extrato hidroetanólico, germinação de esporos, crescimento micelial, controle alternativo

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INTRODUCTION

Target spot and anthracnose are acerola (*Malpighia emarginata* DC.) diseases that threat the leaves and fruit production of this culture. They are caused by two fungi, *Corynespora cassiicola* (Cc) (Berk. & Curtis) and *Colletotrichum gloeosporioides* (Cg) Penz. (Penz. & Sacc.), respectively.

Many fungicides are toxic to humans and other non-target organisms like beneficial crop organisms (Newton et al., 2010). The need for high food production for a constant-growing population on the planet (Koul & Walia, 2009) makes important the control of plant pests and pathogens. Sustainable practices have become important due to the population increase (Woodhouse, 2010) and the common awareness about the need to preserve the environment. Therefore, the study of new antifungal compounds in plants is interesting because plant secondary metabolites are protectors against pathogens and scavengers (Khan & Nasreen, 2010). Furthermore, these compounds could be safer, environmentally friendly and cheaper if compared to synthetic fungicides (Khan & Nasreen, 2010). Moreover, natural products could be researched to become synthetic drugs, including salicylic acid which was found in willow bark (Demain, 2009).

Brazilian flora is very diverse. Brazilian Cerrado is a part of the South American Cerrado. The latter is considered the world's most species-rich tropical savanna (Simon et al., 2009). However, this biome is threatened by agricultural activities such as soybean and *Eucalyptus* plantations (Silva et al., 2010).

Among the ten plant species most cited on the data basis MEDLINE-PubMed, six are native to Brazil and only one of these six plants (*Schinus molle* L.) was studied for antifungal activity, according to Fenner et al. (2006), regardless of the huge variety of Brazilian native plant species that need studies. Indeed, for Brazilian Cerrado plant extracts, there are only a few investigations about this native species with antifungal activity on plant pathogens. Examples of studies on Cerrado plant extracts against phytopathogenic fungi are those from Pereira et al. (2009) and against human fungal pathogens those from Silva Junior et al. (2009; 2010).

The aim of this study was to determine the antifungal activity of aqueous and hydroethanolic extracts from the leaves of 10 plants from the Brazilian Cerrado on *Colletotrichum gloeosporioides* and *Corynespora cassiicola*.

MATERIAL AND METHOD

The study was carried out in the Microbiology and Phytopathology Laboratory at the Faculty of Engineering, Ilha Solteira Campus, São Paulo State University "Júlio de Mesquita Filho" - UNESP, Ilha Solteira, São Paulo State, Brazil.

Plant material

Leaves from ten species were collected in Cerrado areas in the Selviria region, Mato Grosso do Sul State, Brazil: *Aristolochia esperanzae* Kuntze ("jarrinha") voucher no. 5899, *Byrsonima verbascifolia* (L.) DC. ("murici") voucher no. 6052, *Caryocar brasiliense* Cambess. ("pequi") voucher no. 5775, *Casearia sylvestris* Sw. ("café-bravo") voucher no. 3178, *Copaifera langsdorffii* Desf. ("copaíba") voucher no. 4656, *Lafoensia pacari* A. St.-Hil. ("pacari") voucher no. 5586, *Myracrodruon urundeuva* Allemão ("aroeira") voucher no. 5484, *Solanum lycocarpum* A. St.-Hil. ("lobeira") voucher no. 6085, *Stryphnodendron adstringens* (Mart.) Coville ("barbatimão") voucher no. 5993, and *Styrax ferrugineus* Nees & Mart. ("laranjinha-do-cerrado") voucher nº 3031. The voucher specimens are deposited at the Herbarium of the Faculty of Engineering, Ilha Solteira Campus, São Paulo State University "Júlio de Mesquita Filho" - UNESP.

The collected material was taken to the Laboratory where it was washed, dried, ground, and the aqueous and hydroethanolic extracts were prepared (Celoto et al., 2008).

From the acerola leaves with target spot (Cc) and anthracnose (Cg), direct isolation of the phytopathogens was done into Potato-Dextrose-Agar (PDA) medium. Both isolates were preserved in test tubes containing saline solution (Castellani, 1939) and kept at 15ºC. Cg or Cc was transferred again to Petri dishes containing PDA and incubated at 25ºC for seven days.

Mycelial Growth

The effect of extracts on the mycelial growth of Cc and Cg was evaluated with the use of PDA and the extract. The extracts were used at 50% concentration relative to the volume and incorporated in the culture media before autoclaving. Five-millimeter diameter discs were obtained from the fungal colonies and transferred to the center of the Petri dishes containing PDA+extract. The control was constituted of PDA medium only and the PDA disc with the fungal growth. Later the dishes were sealed and kept in an incubator stove at 25ºC. The evaluation was done by measuring the mycelial growth after ten days for Cg and after 13 days for Cc from the experiment installation.

The used experimental design was completely randomized in a factorial scheme with two extractors (water and ethanol), extract of ten plants, two fungi and two controls, totaling 42 treatments and four replicates. Each replicate was a Petri dish. The obtained data were compared to those of the control, and the Mycelial Growth Inhibition Percentage (MGIP) was calculated.

Spore Germination

The effect of the extracts on Cc and Cg spore germination was evaluated at 50% concentration relative to the volume. From the Petri dishes containing PDA and Cg or Cc fungi colony, spore suspensions were prepared as per Celoto et al. (2008). The spore suspensions were gauged at 4 x 10^5 and 4 x 10^4 spores mL^{-1}, respectively, for Cc and Cg. Forty µL of Cg or Cc spore suspension were used and 40 µL of the extract under evaluation or distilled water were pipetted for the control on ELISA (enzyme-linked immunosorbent assay) dish cells. These were put in plastic containers which had at their bottom two sheets of filter paper damped in sterile distilled water. This set was kept in an incubator at 25ºC for 12 hours for Cc and 24 hours for Cg. Then, a drop of lactophenol was added to interrupt spore germination. The counting of 100 spores in each cell was done under an optical microscope, determining the germinated and the non-germinated spores. The germinated spore had a germinative tube equal to or larger than its width.

The used experimental design was similar to the one used for the mycelial growth test, one replicate was represented by a cell on ELISA dish. The data obtained for the treatments were compared to those for the control by calculating the Spore Germination Inhibition Percentage (SGIP).

Both experiments were carried out twice and the mean data of each replicate in the statistical analysis was used. These data underwent analysis of variance through F test by comparing the means through Tukey's test, at 5% probability level.

RESULT

M. urundeuva and *L. pacari* aqueous extracts presented the highest Cc MGIP, 100 and 95%, respectively. They did not differ from each other but differed statistically from the other aqueous extracts, at 5% probability level (Table 1). The other aqueous extracts presented inhibition values below 32%, while *A. esperanzae* and *B. verbascifolia* aqueous extracts stimulated Cc mycelial growth more than the control. As to the hydroethanolic extracts, *M. urundeuva*, *L. pacari and C. brasiliense* inhibited the mycelial growth by 100%, not differing among themselves but differing from the other evaluated extracts. *M. urundeuva* extract was the only one that did not present a statistically significant difference as to the extractor – water or ethanol. This means that either water or ethanol managed to extract antifungal substances, which did not occur for the materials from the other nine plants studied. For all the plant species, numerically, ethanol extracted more antifungal substances than water.

The highest Cc SGIP values, 100, 100, 99, 100 and 98%, were obtained for the aqueous extracts of *S. adstringens*, *C. sylvestris, B. verbascifolia, L. pacari*, and *C. brasiliense,* respectively, differing from the other aqueous extracts but not differing among themselves at 5% probability level. The highest inhibition values for the hydroethanolic extracts were obtained for *M. urundeuva, S. adstringens*, *C. sylvestris*, *S. ferrugineus*, *B. verbascifolia*, *L. pacari* and *C. brasiliense*, all of them with 100% of Cc SGIP and statistically different from the other hydroethanolic

TABLE 1. Mycelial growth inhibition percentage (MGIP) and spore germination inhibition percentage (SGIP) of *Corynespora cassiicola* and *Colletotrichum gloeosporioides* by aqueous (A.E.) and hydroethanolic (H.E.) extracts from the leaves of Cerrado plants at 50% concentration relative to the volume.

Treatment	*Corynespora cassiicola*				*Colletotrichum gloeosporioides*			
	MGIP		SGIP		MGIP		SGIP	
	A.E.	H.E.	A.E.	H.E.	A.E.	H.E.	A.E.	H.E.
Myracrodruon urundeuva	100aA*	100aA	94bA	100aA	95aB*	100aA	93bA	89bB
Stryphnodendron adstringens	12cB	3eA	100aA	100aA	4efA	5fA	100aA	100aA
Casearia sylvestris	12cB	72bA	100aA	100aA	7eB	37cA	100aA	100aA
Copaifera langsdorffii	31bB	64cA	15eB	94bA	2efB	38cA	100aA	100aA
Aristolochia esperanzae	-14†eB	23fA	92bA	89cB	14dB	60bA	100aA	100aA
Styrax ferrugineus	1dB	31eA	75bA	100aA	21cB	36cA	100aA	98aA
Solanum lycocarpum	5dB	29efA	84cA	69dB	1fB	23dA	100aA	70cB
Byrsonima verbascifolia	-34fB	50dA	99aA	100aA	8eB	14eA	100aA	99aA
Lafoensia pacari	95aB	100aA	100aA	100aA	90aB	100aA	100aA	99aA
Caryocar brasiliense	32bB	99aA	98aA	100aA	48bB	63bA	96abA	98aA
C.V.(%)	6,1		1,6		6,4		2,4	

*Means followed by the same letter, uppercase letters on the line and lowercase letters in the column, for each evaluated parameter are not significantly different according to Tukey's test (5%). † There was a stimulus in the mycelial growth compared to the control treatment.

extracts. *M. urundeuva*, *S. adstringens*, *C. sylvestris, B. verbascifolia, L. pacari, and C. brasiliense* aqueous extracts did not statistically differ from their respective hydroethanolic extracts for Cc. This suggests that these two extractor types extract substances that inhibit Cc spore germination at the same proportion.

The highest Cg MGIP was obtained for *M. urundeuva* and *L. pacari* aqueous and hydroethanolic extracts, with minimum above 90%, statistically differing from the other extracts (Table 1). *M. urundeuva* and *L. pacari* hydroethanolic extracts presented statistically higher MGIPs than their respective aqueous extracts, indicating that ethanol extracted more antifungal Cg substances than water.

M. urundeuva aqueous and hydroethanolic extracts and *S. lycocarpum* hydroethanolic extract showed the lowest Cg SGIPs, which differed from that of all the other extracts. The highest Cg SGIPs were noted for the hydroethanolic and aqueous extracts from *S. adstringens*, *C. sylvestris*, *C. langsdorffii*, *A. esperanzae*, *S. ferrugineus*, *B. verbascifolia*, *L. pacari* and *C. brasiliense,* and aqueous extract from *S. lycocarpum,* differing from the other extracts but not differing among themselves. Both water and ethanol were efficient in extracting these plant substances, which inhibited, at the same proportion, Cg spore germination.

L. pacari and *M. urundeuva* leaf extracts presented the highest MGIPs and SGIPs for Cc and Cg isolated from acerola.

DISCUSSION

Among the 20 evaluated extracts, there was a wide variation of antifungal activity. *L. pacari*, *M. urundeuva* and *C. brasiliense* extracts were considered the ones with the highest antifungal activity for both fungi.

Leaf and stem bark hydroethanolic extracts from *L. pacari* presented activity against multiresistant strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Porfírio et al., 2009), which agrees with the antimicrobial activity obtained here for this plant. According to those researchers, the main chemical compounds found are polyphenols, tannins, quinones and alkaloids which may be involved in this activity. Furthermore, Rogerio et al. (2008) verified that stem bark ethanol extract from *L. pacari* and a compound present in this plant, ellagic acid, are effective in suppressing eosinophilic inflammation, which suggests a potential to treat allergies.

L. pacari bark extract has been effective against the yeasts *Candida* spp., *Saccharomyces cerevisiae*, and *Cryptococcus neoformans*; however, no activity against human filamentous fungi or dermathophytes has been reported (Silva Junior et al., 2009; 2010), differing from the antifungal activity found for the filamentous plant pathogens studied here. The ellagic acid was again suggested to be the main active compound of *L. pacari*, associated with cell wall inhibition (Silva Junior et al., 2010). In our study, leaf extracts from this plant had antifungal activity against filamentous fungi. Possibly, the activity found by us could be related to a compound other than ellagic acid. Silva Junior et al. (2010) pointed this compound as antimicrobial, but they used *L. pacari* stem bark whereas we used leaves from this plant.

Lectin was isolated from *M. urundeuva* heartwood extracts and presented activity against phytopathogenic fungi of the *Fusarium* genus and bacteria (Sá et al., 2009a; 2009b). Besides antimicrobial activity, this compound was effective as antioxidant and termite repellent (Sá et al., 2009a). The heartwood of *M. urundeuva* is rich in secondary metabolites (Sá et al., 2009a). This might be connected to some antimicrobial action that the plant develops, indicating the need for further study. In addition, stem bark hydroethanolic extract from *M. urundeuva* was active against bacteria and *Candida* spp. (Alves et al., 2009).

C. brasiliense hydroethanolic leaf extract was more active against Cg from *Psidium guajava* (guava) than from *Xylopia aromatica* (Pereira et al., 2009), which agrees with the antifungal activity reported in our work. *C. brasiliense* fruit extract was also active against bacteria but not against *C. albicans* (Ascari et al., 2010). The same study reported that ethyl gallate was isolated in a high yield from the crude ethanol fruit extract, which suggests that this compound could be responsible for the antimicrobial activity. Again, a different compound may have caused the inhibition of fungi in our work since Ascari et al. (2010) used fruits and we used leaves against Cc and Cg.

The hydroethanolic extracts extracted more antifungal substances in general. Similarly, Celoto et al. (2008) tested 20 medicinal plant species and two extractor types (water and ethanol) and observed that, regardless of the plant species, hydroethanolic extracts caused more inhibition on *Carica papaya* (papaya) Cg mycelial growth. Higher activity for hydroethanolic extracts in comparison to aqueous extracts was also mentioned by Pereira et al. (2009) for *C. brasiliense* and *X. aromatica* against Cg of guava.

Cc and Cg spores manifested themselves more sensitive to the extracts than the mycelial growth when under the action of the studied extracts. This is attributed to the fact that the spores are directly immersed in the extract suspension, whereas the mycelium grew on the medium containing the extract, having thus a more restricted contact with the plant extract than the spores.

Furthermore, the extracts for the spore germination assay were not autoclaved. Autoclaving may cause the loss of some compounds that could have action against the pathogens. Pressure and heat from the autoclaving damage and denature molecules and subcellular structures that lead to the sterilization

of the autoclaved product (Perkins et al., 2004). Autoclaving was chosen in this work because it helps prevent contamination. Celoto et al. (2008) observed that autoclaving modified the antifungal activity of either hydroethanolic or aqueous extracts. This suggests that the Cerrado plants here reported with no or low activity could have the compounds denatured and their action masked by autoclaving. This could be true since *Byrsonima* species were reported to act against *C. albicans* and bacteria (Michelin et al., 2008), and here *B. verbascifolia* is not reported as antimicrobial.

The plants evaluated in this study are not recommended for the control of phytopathogens; however, there are antifungal substances in the studied plants. *M. urundeuva*, *L. pacari* and *C. brasiliense* were the studied plants with better response against both acerola pathogens. As reported in the literature, the found compounds that may have an activity against microorganisms could not be the same compounds responsible for the activity found here. Plants reported here with no effect against the tested fungi could have had their compounds eliminated by autoclaving and the response of inhibition could have been hidden. Further studies should be conducted on the extracts from all the plants tested here, including the comparison of activity among different parts of the plants, toxicological assays, analysis of substances responsible for the activity, and field assays to evaluate the effect of plant extracts on the disease.

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