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Protection of *Sideroxylon obtusifolium* seeds against *Colletotrichum* sp. with *Caesalpinia ferrea* extract

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***Sideroxylon obtusifolium* (Roem. & Schul.) Penn. is a native species from Caatinga biome, but due to disorderly exploitation for phytoteraphy industry, it is in danger of extinction. Recent researches report that the diversity of vegetal species in Brazilian semiarid regions, when meticulously assessed through methods that focus on properties of molecules from different plant structures, may present a high potential for the discovery and development of new antifungal substances. The aim of this work was to evaluate the effects of *S. obtusifolium* seeds treatment with *Caesalpinia ferrea* extract on the control of *Colletotrichum* sp. In each treatment, 100 seeds were inoculated with the pathogen through immersion in a suspension of *Colletotrichum* sp. conidia, and then subjected to the following treatments: Seeds without treatment and not inoculated (T_1), seeds infected with *Colletotrichum* sp. (T_2), infected seeds treated with captan fungicide (T_3) and infected seeds treated with *C. ferrea* extract (T_4). *C. ferrea* extract provided a higher protection to *S. obtusifolium* seeds and seedlings against *Colletotrichum sp*., indicating that it is a viable and sustainable biotechnological resource against pathogens and a promising molecule for the development of new antifungal substances.**

Key words: Antifungal activity, native species, alternative control.

INTRODUCTION

Sideroxylon obtusifolium (Roem. & Schult.) Penn is a native forestry species from Caatinga biome (Silva and Dantas, 2013). It as non-cultivated fruit tree, popularly known by local inhabitants as quixabeira, quixaba,

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sapotiaba or coronilha (Silva et al., 2012) and it is exploited in popular medicine and for industrial production of phytotherapeutic drugs (Gomes et al., 2010). This way, the development of forest recovery programs through the production of seedlings from high quality seeds and with genetic variance is necessary (Beltrão et al., 2008; Vechiato and Parisi, 2013). For this purpose, the sanitary and physiological quality of seeds is highly important, as it will determine the development of healthy seedlings in nurseries (Mondego et al., 2014).

Besides damaging the germination potential, pathogens use seeds as a dissemination vehicle and a survival shelter, involving these reproductive structures in the continuity of the biological cycle of the disease through the generations of the host plant. Thereby, researches exploring the transmission from seeds to seedlings contribute to the advancement of disease management strategies, showing that the initial inoculum of the causal agent reached the infected area through seeds or other ways of early fungal infection (Poletto et al., 2014).

Among the fungi related to seeds, there are currently 59 species of *Colletotrichum* in Brazil, infecting about 154 host plants, like several native forestry species such as *Lithraea brasiliens* March., *Myracrodrun urundeuva* Fr. All, *Tabebuia impetiginosa* (Mart. ex DC.) Standl., *Apeiba tibourbou* Aubl. and *Dalbergia nigra* (Vell.), causing seed rot, leaf spots, low germination and damages at seedling development (Vechiato and Parisi, 2013).

Nevertheless, there is still lack of studies on forestry seeds and efficiency of chemical and alternative phytosanitary products designated for the establishment of sanitary protocols for seedlings production (Mertz et al., 2009). Recent researches report that diversity of vegetal species on Brazilian semiarid region, when assessed with methods that focus on the action of molecules present in different tissues of the plant, might constitute a valuable tool for discovery of new antifungal

Among vegetal species with antifungal properties, *Caesalpinia ferrea* Mart. Ex. Tul. stand out as one of the most studied for its compounds and biological and chemical activities (Ferreira and Soares 2015). Several studies on *C. ferrea* extracts and isolated compounds with antimicrobial characteristics have been performed to control fungi such as *Aspergillus niger*, *Trichoderma viride* and *Penicillium cyclopium* (Marreiro et al., 2014; Martins et al., 2014).

New studies are still necessary to discover new bioactive molecules with highly efficient pathogen control, as it is considered more complex for pathogens to develop resistance against products derived from plant extracts due to their wide chemical constitution and modes of action (Ferreira et al., 2013). Besides that, considering the importance of seed quality and sanity for crop yield and the great potential of *S. obstusifolium* for reforestation and pharmaceutical industry (Oliveira et al., 2012), this work was carried out with the objective to evaluate the efficacy of *C. ferrea* extract in *Colletotrichum* sp. infection in *Sideroxylon obtusifolium* (Roem. & Schult.) Penn. seeds.

MATERIALS AND METHODS

Seeds obtainment

S. obtusifolium seeds were extracted from mature fruits from ten matrix trees located in Boa Vista, Paraiba State, in the first fortnight of February 2013. Trees were situated at an average altitude of 490 m and at the following coordinates: M1 6°57'6,65" S, 35°43'4,60" W; M2 6°57'6,91" S, 35°43'4,68" W; M3 6°57'6,70" S, 35°43'48,7" W; M4 6°57'6,82" S, 35°43'48,6" W; M5 6°57'7,01" S, 35°43'4,79" W; M6 6°57'6,78" S, 35°43'5,05" W; M7 6°57'6,95" S, 35°43'5,04" W; M8 6°57'7,14" S, 35° 43,48,3" W; M9 6°57'7,18" S, 35°43'46,8" W; M10 6°57'7,32" S and 35°43'44,4" W. After harvest, fruits were packed in polyethylene bags and taken to the Seed Analysis Laboratory of Federal University of Paraiba. Seeds were extracted by the natural fermentation method for 72 h, followed by washing in running water and dried on paper towel at room temperature (25 ± 2°C) (Silva et al., 2012).

Water content

Water content was determined with an oven at 105°C for 24 h (Brasil, 2009), considering four replicates of 25 seeds per matrix tree.

Inoculum and aqueous extract obtainment

Samples from each seed lots were analyzed for determination of the presence of microflora, using four replicates of 25 seeds per treatment. Seeds were surface disinfected through immersion in sodium hypochlorite (2%) for 5 min and then distributed on Petri dishes with sterilized and humidified filter paper. After a seven-day incubation period at room temperature (25 ± 2°C), fungal structures were analyzed with an optical and a stereoscopic microscope. Identification of the species was performed with an identification key (Barnett and Hunter, 1972). Then, the obtainment of *Colletotrichum* sp. isolates was proceeded following methodology proposed by Medeiros et al. (2013), incubating seeds on Petri dishes containing sterilized PDA growth media (1000 ml of deionized water, 200 g of potato, 20 g of dextrose and 17 g of agar).

Caesalpinia ferrea leaves were used to produce the aqueous extracts. 500 g of fresh weight were immersed for 5 min in sodium hypochlorite solution (2%) and then dried at room temperature (25 ± 2°C) for 24 h. After this period, leaves were kept in an oven with forced air circulation at 40 ± 2°C, until stabilization of residual moisture, and then pulverized in a knife mill to obtain plant raw material (PRM) (Stange et al., 2009).

PRM was diluted in deionized water in a 1:3 proportion (Pedroso et al., 2011), and later, the suspension was subjected to constant agitation at 35°C for 24 h. Then, the concentrated was obtained through vacuum filtration and subsequently lyophilized (Mot et al., 2012) to turn material into powder for conservation in a freezer at -20°C.

Seed inoculation and treatment

Fungal suspension concentration was determined with Neubauer chamber, resulting in approximately 1 x 10^5 *Colletotrichum* sp.

Table 1. Germination rate (G) and germination speed index (SGI) of *Sideroxylon obtusifolium* seeds inoculated with *Colletotrichum* sp.

Matrix	G (%)		GSI	
	T1	T2	T1	T2
1	95^{aA}	79^{aB}	1.48^{aA}	0.55^{bB}
2	90^{aA}	62^{bB}	1.35^{aA}	0.71^{aB}
3	93^{aA}	10d^{B}	1.43^{aA}	0.20d^{B}
4	92^{aA}	57^{bB}	1.46^{aA}	0.35c^{B}
5	88^{aA}	38c^{B}	1.38^{aA}	0.35c^{B}
6	85^{aA}	45c^{B}	1.44^{aA}	0.55^{bB}
7	53c^{A}	8d^{B}	0.72^{bA}	0.12d^{B}
8	50c^{A}	6d^{B}	0.64^{bA}	0.14d^{B}
9	42d^{A}	6d^{B}	0.58^{bA}	0.10d^{B}
10	24e^{A}	5d^{B}	0.57^{bA}	0.10d^{B}
CV (%)	9.8			

*****Means followed by the same letter, lower case in columns and upper case in rows, do not significantly differ from each other by Scott-Knot test ($p<0.05$). T1 = non inoculated seeds; T2 = *Colletotrichum* sp. inoculated seeds.

conidia mL^{-1}. The treatments consisted of seeds without inoculation and treatment (T_1), inoculated seeds (check) (T_2), inoculated seeds treated with Captan fungicide (positive control) (T_3) and inoculated seeds treated with *C. ferrea* extract (T_4).

At room temperature, seeds were disinfected with sodium hypochlorite (2%) for 2 min, ethanol 70% for 30 s, washed two times with deionized water and dried with towel paper for 30 min (Ferraz and Calvi, 2010). Inoculation with the pathogen was performed through seed immersion in 20 mL of fungal suspension for 12 h. After inoculation, seeds were immersed for 24 h in *C. ferrea* extract solutions with concentrations of 0.075, 0.15; 0.31 and 0.62 mg mL^{-1}, as described by Flavio et al. (2014). For the positive check treatment (T_3), application of the fungicide was also after the pathogen inoculation (Mondego et al., 2014).

Physiological and sanitary quality determinations

Evaluation of the physiological potential was performed with seeds from T_1 and T_2 treatments, and for T_3 and T_4 treatments, the incidence and transmissivity rate of *Colletotrichum* sp. was also determined.

Seeds were manually scarified with sandpaper no. 80 at the opposite side of the hilum and 2 cm depth sowed in transparent plastic boxes (11.0 cm x 11.0 cm x 3.5 cm), previously disinfected with sodium hypochlorite (2%), filled with sterilized vermiculite (Silva et al., 2012) and moistened with deionized water until 60% of its water holding capacity (BRASIL, 2009). Boxes were maintained in germination chamber at 30°C with a 12 h photoperiod, equipped with fluorescent lamps (4 x 20 W).

Evaluations were carried out on alternative days, from the 15th to 30th day after sowing, with standard seedlings emergence, and the results were expressed in percentage. Seed germination rate and germination speed index (GSI) were determined according to Brasil Ministério da Agricultura, Pecuária e Abastecimento (2009) and Maguire (1962), respectively.

On the 30th day, pathogen transmissivity was assessed. Over this period, diseased seedlings with symptoms of *Colletotrichum* sp. infection in cotyledons, roots, stems and leaves and rot seeds were observed (Auer and Álvaro, 2010). To confirm pathogen etiology, seeds and seedlings shoot and root fragments were sectioned, disinfected and incubated in Petri dishes, following methodology proposed by Ferraz and Calvi (2010) and Medeiros et al. (2013), respectively. After fungal culture development, the identification was executed following an identification key of Barnett and Hunter (1972). Fungus transmission rate to seedlings was calculated using the equation (TR (%) = [IR (%)/DI(%)] × 100), adapted from Teixeira and Machado (2003), where IR = infection rate in seedlings with symptoms of *Colletotrichum* sp.; DI = disease incidence in seeds artificially inoculated. Survival rate (SR) was also calculated, according to the equation adapted from Teixeira and Machado. (2003), where SR (%) = [No. of germinated seedlings – No. of infected seeds/total of seeds] × 100).

Experimental design and statistical analysis

The experimental design was completely randomized, with treatments distributed in a 10 × 7 factorial layout, with ten matrix trees and six seed treatments (plus check). Data were subjected to variance analysis (ANOVA), regression study and comparison of means by Scott-Knot test ($p<0.05$). For variables that fit in the regression quadratic model, the higher and/or lower values were determined by derivation of the equation.

RESULTS AND DISCUSSION

A wide range of fungi genera was present in the *S. obtusifolium* seeds microbiota, which included *Aspergillus niger*, *Aspergillus flavus*, *Aspergillus* sp., *Botrytis* sp., *Colletotrichum* sp., *Chaetonium* sp., *Cladosporium* sp., *Curvulária* sp., *Fusarium* sp., *Helminthosporium* sp., *Nigrospora* sp. and *Penicillium* sp. This is the first report on the microflora associated with this forestry species.

Evaluating the deleterious effects of *Colletotrichum* sp. due to its inoculation on *S. obtusifolium* seeds (Table 1), higher germination rates and GSI values were found in non-inoculated seeds (T1, related to all matrices, except 7, 8, 9 and 10). Meanwhile, the lowest germination

Figure 1. *Sideroxylon obtusifolium* seedling infected with *Colletotrichum* sp.

Table 2. Adjusted models through regression analysis for *Colletotrichum* sp. incidence (%) in *Sideroxylum obtusifolium* seeds artificially inoculated and treated with *Caesalpinia ferrea* extract and fungicide.

Matrix	Adjusted regression equations	R^2
1	$y = 459.92x^2 - 366.45x + 52.747$	0.65
2	$y = 371.71x^2 - 296.17x + 42.631$	0.65
3	$y = 393.83x^2 - 305.63x + 52.352$	0.56
4	$y = 504.02x^2 - 401.59x + 57.805$	0.65
5	$y = 486.76*x^2 - 390.15x + 57.115$	0.69*
6	$y = 424.72x^2 - 343.44x + 52.408$	0.60
7	$y = 376.23*x^2 - 314.20x + 52.436$	0.69*
8	$y = 500.58x^2 - 384.97x + 54.469$	0.65
9	$y = 422.12x^2 - 336.33x + 48.412$	0.65
10	$y = 485.12x^2 - 386.53x + 55.637$	0.65

R^2 = coefficient of determination. *Significant through Scott-Knot test (P <0.05).

performance was associated with this same lot of seeds (7, 8, 9 and 10), although they did not statistically differ from seeds from matrix 3.

Vechiato and Parisi (2013) reported that *Colletotrichum* sp., with low or mild incidence on forestry species seeds is a potentially pathogenic fungus; however, according to these authors, it is not possible to affirm the damages this genus may cause. However, the data found in this work indicates that *Colletotrichum* sp. has a negative interference on *S. obtusifolium* seed germination, decreasing the speed and the number of emerged seedlings. These results were confirmed by observed symptoms such as necrotic lesions on cotyledons, young leaves, roots, followed by seedlings damping off (Figure 1).

These results corroborate Lopes et al. (2011), who reported that fungal infection in angico branco (*Anadenanthera colubrina* (Vell.) Brenan.) seeds drastically affected their physiological quality, even completely inhibiting germination in some cases. Santos et al. (2001) also found similar results assessing the influence of *Colletotrichum* sp. on canafistula (*Peltophorum dubium* (Sprengel) Taubert.) seeds, also Auer and Álvaro (2010) on araucaria (*Araucaria angustifólia* (Bertol.) Kuntze.) seedlings development, indicating the progression of early lesions on cotyledons to stem strangulation and seedling death.

With regards to the incidence of *Colletotrichum* sp. in *S. obtusifolium* seeds inoculated and treated with *C. ferrea* extract or captan, all the matrices fit a quadratic model (Table 2), in which a higher incidence of the pathogen is verified on check treatment (0 mg/mL) (Figure 2). However, even in the lowest extract doses (0.075 mg mL^{-1}), the pathogen incidence decreased by 92 and 96% in matrices 7 and 5, respectively, whereas the other seeds lots presented an absolute control of the fungus. Notwithstanding, the chemical treatment with fungicide reduced the occurrence of infesting microorganisms without eradicating them, with an average incidence of 69.9% among the matrices, a value indeed superior than those observed for matrices 2 and 9 without *C. ferrea* extract (Figure 2).

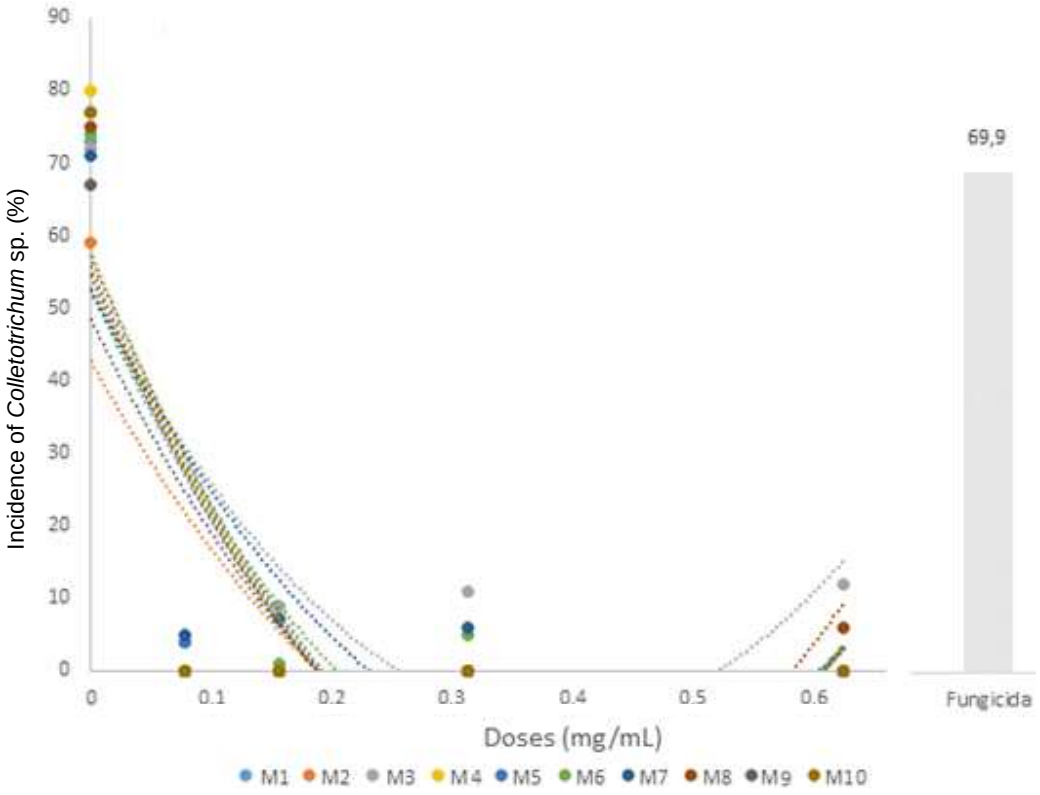

Figure 2. Incidence of *Colletotrichum* sp. in *S. obtusifolium* seeds inoculated and treated with *Caesalpinia ferrea* extract and captan fungicide.

The high fungal incidence in seeds without treatment might be due to the incubation conditions throughout the germination test which are optimal for fungi development, as in natural conditions, this pathogen occurs as low to mild incidence on forestry species (Vechiato and Parisi, 2013). According to these authors, the high occurrence of *Colletotrichum* sp. in *S. obtusifolium* matrices confirms the affinity of this pathogen for forestry species, endorsing their observations with aroeira-brava (*Lithraea brasiliens* March.), aroeira (*Myracrodrun urundeuva* Fr. All.), ipê-roxo (*Tabebuia impetiginosa* (Mart. ex DC.) Standl.), pau-de-jangada (*Apeiba tibourbou* Aubl.), cedar (*Cedrela fissilis* Vell.) and jacarandá-da-baía (*Dalbergia nigra* (Vell.).

Treatment with different doses of *C. ferrea* extract promoted significant decrease in the incidence of *Colletotrichum* sp. in *S. obtusifolium* seeds. Similar results were obtained by Lazarotto et al. (2009), who reported a reduced incidence of this fungus in cedar (*Cedrela fissilis* Vell.) seeds treated with garlic (*Allium sativum*) and boldo-brasileiro (*Plectranthus barbatus*) extracts. In seeds of other forestry species, such as amendoim-bravo (*Pterogyne nitens* Tul.) (Medeiros et al., 2013), sansão-do-campo (*Mimosa. caesalpiniaefolia* Benth.) (Leite et al., 2012) and flamboyant-mirim (*Caesalpinia pulcherrima* L.) (Medeiros et al., 2011), the adoption of vegetal extracts was also proven as an efficient tool to control seed pathogens.

The transmissivity rate (Figure 3) was negatively affected by moderate doses of *C. ferrea* extract in matrices 5, 8 and 12, which with the quadratic model, presented an initial increase of this parameter as compared to check treatment, with maximum values at the doses of 0.35, 0.30 and 0.21, respectively. Although it decreased reasonably from this point, even reaching complete suppression of transmissivity for matrices 8 and 12 (Table 3). This way, only doses higher than 0.5 mg L^{-1} for matrix 12 and the maximum evaluated dose (0.62 mg mL^{-1}) for matrix 8 were considered effective against transmission of the disease from seeds to seedlings of *S. obtusifolium*.

The other matrices likewise fit the quadratic model (Table 3). Almost all of them had a lower transmissivity rate following the increase of the extract doses, apart from matrix 13, which unexpectedly had this parameter enhanced with higher dose extracts. Fungicide treatment resulted in an average transmissivity of 20.4%, a value inferior only to matrices 2, 3, 12 and 15 without sanitary treatments (Figure 3).

Local penetration through testa is the most common mechanism for necrotrophic microorganisms, as *Colletotrichum* sp., to start their cycle (Poletto et al.,

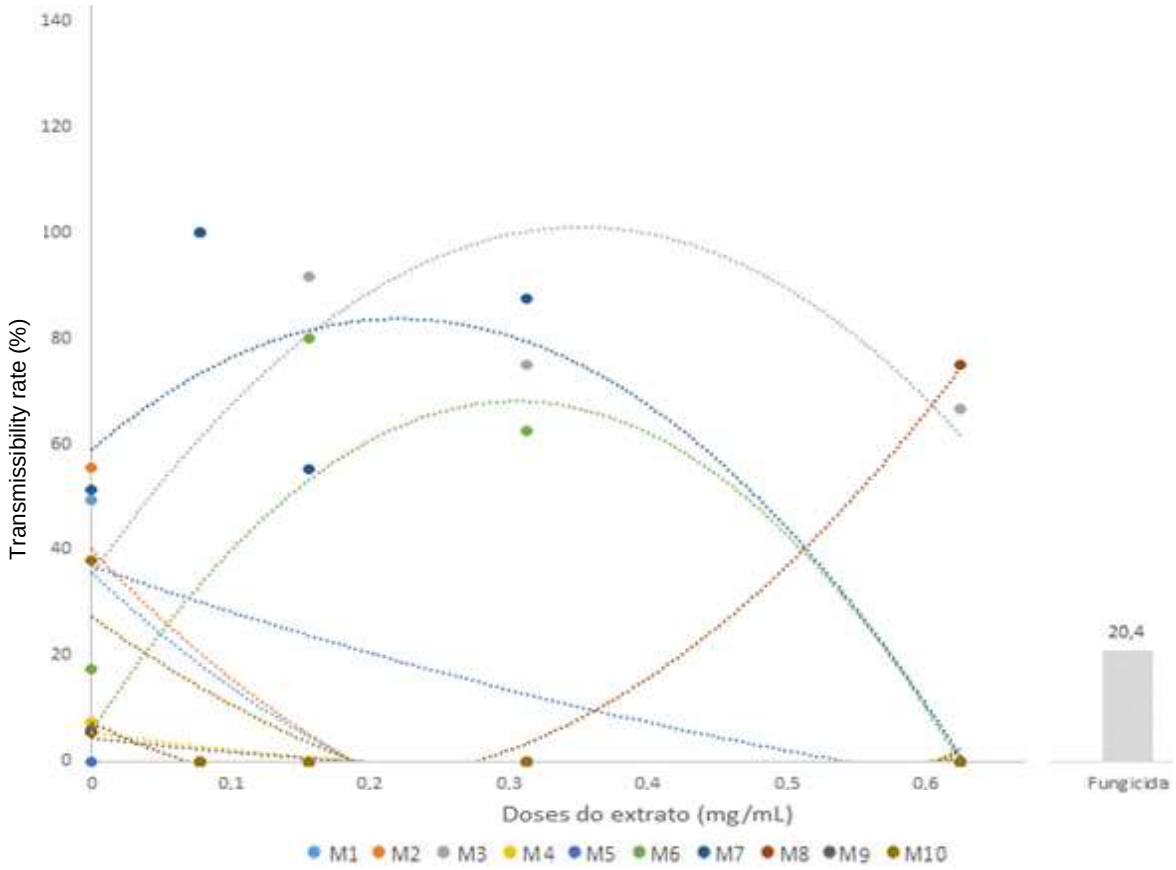

Figure 3. Transmissibility rate (%) of *Colletotrichum* sp. in seedlings of *S. obtusifolium* from inoculated seeds and treated with extract of *C. ferrea* and captana fungicide.

Table 3. Adjusted models through regression analysis for *Colletotrichum* sp. transmissivity rate (%) in *S. obtusifolium* seeds artificially inoculated and treated with *C. ferrea* extract.

Matrix	Adjusted regression equation	R^2
1	$y = 311.48x^2 - 248.18x + 35.724$	0.65
2	$y = 349.13x^2 - 278.18x + 40.041$	0.65
3	$y = - 530.39x^2 + 373.68x + 35.244$	0.42
4	$y = 47.252x^2 - 37.649x + 5.4192$	0.65
5	$y = 41.03x^2 - 90.444x + 36.934$	0.12*
6	$y = - 669.87x^2 + 409.45x + 5.529$	0.63
7	$y = - 507.38x^2 + 224.05x + 58.962$	0.74*
8	$y = 384.47x^2 - 132.94x + 7.335$	0.99
9	$y = 37.991x^2 - 30.27x + 4.3571$	0.65
10	$y = 238.95x^2 - 190.39x + 27.405$	0.65

R^2 = Coefficient of determination. *Significant through Scott-Knot test (P <0.05).

2014); this way, pathogens associated with seeds are spread by infection (establishment in internal tissues) or infestation (passive contamination on testa) of these reproductive structures. Thus, fungal transmission rate depends on the amount and localization of the inoculum in seeds (Sá et al., 2011). Due to this, the inoculation period adopted in this work might have provoked the dissemination of the pathogen to seed embryo tissues. As a result, it was observed that during *S. obtusifolium* germination, the biological cycle of *Colletotrichum* sp.

might have developed, with its spores contaminating various seedling structures, justifying the considerable transmissivity rate even in treated seeds.

Several authors have described antifungal properties of *C. ferrea* extract derived from different plant organs. However, successful results were only reported for extracts obtained from fruits (Zanin et al., 2012), seeds (Cavalheiro et al., 2009) and bark (Ferreira and Soares, 2015), and this work is the first study on the potential of leaves extract for disease control. It is also noticeable that the obtained results confirm those of Ferreira and Soares (2015), who observed antifungal properties of extracts from this species bark against *Colletotrichum lindemuthianum* and *C. truncatum*. Yet, further studies are required to elucidate *C. ferrea* potential as raw material for antifungal aqueous extracts or as a source of bioactive molecules with biotechnological relevance and environmental safety and sustainability.

Conclusion

Coletrotichum sp. infection severely inhibited germination rate and speed in almost all *S. obtusifolium* matrices, except lots 1 and 2, which demonstrated resistance to this pathogen. *C. ferrea* extract was effective in concentrations above 0.5 mg mL^{-1}, reducing both incidence and transmissivity rate of *Colletotrichum* sp. in *S. obtusifolium* seeds.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

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