

Antagonism of Plant Pathogens by *Calotropis procera*

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Abstract

Phomopsis sojae and *Sclerotinia sclerotiorum* are responsible for stem and pod dryness and white mold in soybean. These pathologies directly affect the quality of seeds/grains and compromise the entire plant. The use of extracts from different plants has been the subject of research for the control of several phytopathogens. *Calotropis procera* is among botanical species that synthesize efficient compounds for biocontrol. In this context, the aim of this study was to evaluate the *in vitro* effect of *C. procera* aqueous extract on *P. sojae* and *S. sclerotiorum*. The experiment was carried out in completely randomized blocks in a 2 × 5 factorial scheme (two fungi and five extract concentrations 0%, 5%, 10%, 15% and 20%) with 4 replicates. *C. procera* aqueous extract concentrations were added to Petri dishes containing PDA. After 48 hours, the mycelial growth rate was evaluated. After seven days of incubation, the fungal colony area, sporulation, and germination of *P. sojae* and *S. sclerotiorum* were evaluated. There was significant interaction between fungi × extract concentrations ($p < 0.05$) for all variables analyzed. The mycelial growth rate of *P. sojae* was lower than that of *S. sclerotiorum*. The diameter of the *P. sojae* fungal colony was smaller than that of *S. sclerotiorum* when concentrations of 5%, 10% and 15% were used. As the extract concentration increased, fungi sporulation and germination reduced.

Keywords: stem and pod dryness, white mold, silk cotton, plant extracts

1. Introduction

Soybeans (*Glycine max* L.) belongs to the Fabaceae family and is an annual cycle plant with determined, indeterminate and semi-determined growth habit. It is one of the main oilseeds grown worldwide (Oliveira & Hecht, 2016). Soy is rich in protein and oil, 40% and 22%, respectively (Pratap et al., 2016), which makes it an important raw material for human and animal nutrition (Voora et al., 2020), currently being one of the most traded commodities, with numerous uses (SOYSTAT, 2016). In 2020, Brazil produced about 133 million tons, followed by the USA (116 million tons), Argentina (53 million tons) and China (17 million tons), being the world's largest producer (FAO, 2020).

Several plant pathogenic fungi (Hosseini et al., 2020; Haddad et al., 2017) affect soybean crop, among which *Phomopsis sojae* and *Sclerotinia sclerotiorum* stand out. These phytopathogens cause dry stem (Hosseini et al., 2020) and white mold (Pawlowski et al., 2019) respectively. *P. sojae* causes production losses by affecting seeds/grains (Mena et al., 2020). *S. sclerotiorum* attacks the entire plant by depositing its mycelium (Ranjan et al., 2018). It is known that diseases are one of the factors that limit the production of many plants worldwide (Kumar et al., 2016). The use of agrochemicals to control these two fungi in soybeans is an effective and successful approach (Willbur et al., 2019). However, the large-scale use of agrochemicals has resulted in problems such as environmental pollution, decreased biodiversity, and resistance to pathogens, among others (Haq et al., 2020).

Sustainable management systems are essential tools to maintain yield over the years. In these systems, plants can be protected from disease with environmentally friendly tools, low impact on production and environment (Acosta-Motos et al., 2020). Plants produce several metabolites that can act against phytopathogens (Mishra &

Arora, 2018). The use of plant extracts to control phytopathogens has been widely studied (Shuping & Eloff, 2017; Palou et al., 2016; Zaker et al., 2016). Plant extracts have been proven to be rich in bioactive compounds and antioxidants (Choudhury et al., 2018). The advantage of using natural extracts is that they can be prepared by the farmer himself as an alternative in the management of diseases (Isman, 2017).

The botanical species *Calotropis procera*, also known as silk cotton, is a plant with wide geographical distribution (Yoganandam et al., 2019). It contains bioactive compounds such as phenols, polysaccharide terpenes and flavonoids (Gawade et al., 2017). Due to its numerous antifungal properties (Khan et al., 2019), the species has been extensively studied (Mohamed et al., 2017; Cavalcante et al., 2016). Ali et al. (2020) found that *C. procera* inhibits the *in vitro* mycelial growth of *Alternaria alternata*. Unlike chemical botanical fungicides, offer better protection for crop, soil and environment. In view of the need for alternative control of these phytopathogens and the possibility of biocontrol by *C. procera*, the aim of this study was to evaluate the effect of different *C. procera* aqueous extract concentrations on *P. sojae* and *S. sclerotiorum*.

2. Method

The experiment was carried out at the Laboratory of Phytopathology, State University of Montes Claros, Janaúba Campus, Minas Gerais. The Laboratory of Biotechnology and Fungi Genetics, Federal University of Lavras provided *P. sojae* and *S. sclerotiorum* strains. To carry out tests, fungi were grown in PDA medium (Potato, Dextrose, Agar) for seven days at room temperature in continuous dark. *C. procera* leaves were collected in the first morning hours, which were washed under running water and dried on paper towels. Subsequently, 500 grams of leaves were weighed and submitted to disinfection with sodium hypochlorite (1%, 30 seconds), 70% alcohol (30 seconds) and triple washing with distilled water.

2.1 Preparation of *Calotropis procera* Aqueous Extract

C. procera leaves were cut and crushed in blender with 500 milliliters of distilled water. Then, in laminar flow chamber, the extract was filtered with sterile gauze and filter paper. Then, the liquid was transferred to airtight glass vials and autoclaved at 121 °C for 20 minutes. The autoclaved extract was deposited in sterile plastic tubes to be centrifuged at 13,000 rpm for 15 minutes. The supernatant was used in tests.

2.2 *In vitro* effect of *Calotropis procera* Aqueous Extract on *Phomopsis sojae* and *Sclerotinia sclerotiorum*

PDA culture medium was prepared, which was added of *C. procera* aqueous extract (50% v/v) so that the final concentrations in Petri dishes were 5%, 10%, 15% and 20%. In addition, control plates were prepared, that is, PDA medium without the addition of extract. Discs of 3 mm of edge of *P. sojae* and *S. sclerotiorum* cultures grown for seven days were transferred to the center of a 7-cm petri dish.

Discs were kept at room temperature in continuous dark. After 48 hours, the mycelial growth rate was evaluated and after seven days, mycelial growth, fungi sporulation and germination were evaluated. Mycelial growth was measured with the aid of millimeter rule for two days in two perpendicular directions marked at the bottom of each Petri dish. The mycelial growth rate was calculated using formula proposed by Silva et al. (2015) in which there is proportion of mycelial growth subtraction of 48 for 24 hours by subtracting the longest time for the shortest time (48-24). Results were expressed in mm d⁻¹.

2.3 Effect of *Calotropis procera* Aqueous Extract on the Mycelial Growth, Sporulation and Germination of *Phomopsis sojae* and *Sclerotinia sclerotiorum*

After seven days of incubation, the diameter of *P. sojae* and *S. sclerotiorum* colonies submitted to different *C. procera* concentrations was measured with the aid of millimeter rule. Ten milliliters of sterile distilled water plus 0.08% Tween were deposited on plates containing fungal colonies. Spores were placed in suspension with the aid of glass slide. Subsequently, 3 drops of lactophenol were added to the suspension for spore count in Neubauer chamber under optical microscope. To evaluate spore germination, 100 µL of the previously obtained suspension (before applying lactophenol) were transferred to Petri dishes containing agar-water medium and stored at room temperature for 12 hours. After that period, 100 germinated and non-germinated spores were quantified under optical microscope, being considered germinated the spore with germ tube greater than or equal to the spore length.

2.4 Experimental Design and Statistical Analysis

The experiment was carried out in a 2 × 5 factorial scheme, with two fungi and five extract concentrations (0%, 5%, 10%, 15% and 20%), with four replicates. Data were submitted to analysis of variance at 5% probability by the F test. In case of significant interaction (p < 0.05), data were unfolded. The averages of the two fungi were submitted to the F test and extract concentrations were submitted to regression analysis, choosing the equation

that best described the data behavior and with the highest determination coefficient (R^2). Analyses were performed using the R software version 3.5 (R Core Team, 2020).

3. Results

The result of the analysis of variance is shown in table 1. Significant interaction was observed between fungi and extract concentrations ($p < 0.05$) for all variables analyzed. All determination coefficients (R^2) of equations were greater than 60%.

Table 1. Summary of the analysis of variance, response variables: Mycelial growth rate, colony diameter, spore germination and sporulation of *P. sojae* and *S. sclerotiorum* as a function of different *C. procera* aqueous extract concentrations

Variation source	Medium Square			
	Mycelial growth rate	Colony diameter	Spore germination	Sporulation
Fungi	0.0038*	1.02*	864.90*	15,444.90*
Concentrations	0.0015*	0.47*	2016.56*	2,333.78
Fungi × Concentrations	0.0026*	0.71*	149.21*	222.83*
Experimental error	0.0000	0.05	23.78*	176.23*
Coefficient of variation (%)	0.43	3.63	5.79	17.87

Note. * Significant at 5% probability by the F test.

Regarding the interaction of fungi within each concentration level, it was found that at concentrations of 0%, 5%, 10% and 15%, the mycelial growth rate of *P. sojae* was lower than that of *S. sclerotiorum* (Table 2). In contrast, at concentration of 20%, the mycelial growth rate of *S. sclerotiorum* was lower (Table 2). Regarding the interaction of different concentrations within each fungus level, quadratic behavior for *P. sojae* was verified. Deriving the equation, minimum point of 9.33% was found, that is, the lowest mycelial growth rate would be verified at this concentration. The mycelial growth rate of *S. sclerotiorum* showed linear behavior, and as the extract concentration increased, the mycelial growth rate decreased (Table 3).

Table 2. Unfolding of the fungal species interaction within each concentration level of the extract of *C. procera*. Variable answer: Mycelial growth rate (mm d^{-1})

Fungi	Concentrations of the aqueous extract of <i>C. procera</i>				
	0%	5%	10%	15%	20%
<i>P. sojae</i>	0.3735a	0.3496a	0.3586a	0.3395a	0.3782b
<i>S. sclerotiorum</i>	0.4062b	0.4020b	0.3812b	0.3693b	0.3365a
Minimal significant difference	0.002				

Note. Means followed by the same letter in the column do not differ by the F test at 5% probability.

Table 3. Unfolding of the interaction concentrations of *C. procera* extract within each level of *procera* fungus species. Variable answer: Mycelial growth rate (mm d^{-1})

Fungi	Equation	R^2
<i>P. sojae</i>	$y = 0.3739* - 0.0056*x + 0.0003*x^2$	64.53%
<i>S. sclerotiorum</i>	$y = 0.4135* - 0.0034*x$	93.32%

Note. * Significant at 5% probability by the t test.

The diameter of the *P. sojae* fungal colony was smaller than that of *S. sclerotiorum* when concentrations of 5%, 10% and 15% were used, whereas at concentration of 20%, the extract efficiency against *S. sclerotiorum* was greater (Table 4). Evaluating the diameter within each extract concentration, quadratic behavior was observed for *P. sojae*. Deriving the equation, minimum point of 10.05% was found, a value where the smallest colony diameter would be found (Table 5). On the other hand, the behavior of *S. sclerotiorum* was linear, reducing the diameter as more extract was added to the medium (Table 5).

Table 4. Unfolding of the fungal species interaction within each concentration level of the extract of *C. procera*. Variable answer: Diameter (cm)

Fungi	Concentrations of the aqueous extract of <i>C. procera</i>				
	0%	5%	10%	15%	20%
<i>P. sojae</i>	6.27a	5.87a	6.02a	5.70a	6.35a
<i>S. sclerotiorum</i>	6.82b	6.75b	6.75b	6.20b	5.65b
Minimal significant difference	0.32				

Note. Means followed by the same letter in the column do not differ by the F test at 5% probability.

Table 5. Unfolding of the interaction concentrations of *C. procera* extract within each level of procera fungus species. Variable answer: Diameter (cm)

Fungi	Equation	R ²
<i>P. sojae</i>	$y = 6.282143* - 0.093357*x + 0.004643*x^2$	64.12%
<i>S. sclerotiorum</i>	$y = 6.945* - 0.058*x$	93.50%

Note. * Significant at 5% probability by the t test.

When assessing the action of the aqueous extract on the sporulation of both fungi, it was observed that at all concentrations used, the effect was greater on *S. sclerotiorum* (Table 6). It was observed that the percentage of action of the extract on *S. sclerotiorum* was 30%, 34%, 42%, 41% and 52% higher than on *P. sojae*. The sporulation of both fungi showed linear behavior (Table 6). As the extract concentration increased by one unit, 2.21 *S. sclerotiorum* sporulation units were reduced (Table 7). For *P. sojae*, as the extract concentration increased by one unit, 2.02 sporulation units were reduced.

Table 6. Unfolding of the fungal species interaction within each concentration level of the extract of *C. procera*. Variable answer: Sporulation ($\times 10^5$)

Fungi	Concentrations of the aqueous extract of <i>C. procera</i>				
	0%	5%	10%	15%	20%
<i>P. sojae</i>	107.50b	107.51b	102.25b	83.75b	68.75b
<i>S. sclerotiorum</i>	76.50a	71.00a	58.50a	49.00a	32.50a
Minimal significant difference	15.20				

Note. Means followed by the same letter in the column do not differ by the F test at 5% probability.

Table 7. Unfolding of the interaction concentrations of *C. procera* extract within each level of procera fungus species. Variable answer: Sporulation ($\times 10^5$)

Fungi	Equation	R ²
<i>P. sojae</i>	$y = 114.20* - 0.25*x$	87.23%
<i>S. sclerotiorum</i>	$y = 79.55* - 2.21*x$	97.53%

Note. * Significant at 5% probability by the t test.

The germination of *P. sojae* and *S. sclerotiorum* spores was negatively affected by the highest extract concentrations. The extract was more efficient in controlling *S. sclerotiorum* at concentrations of 10% and 20% (Table 8). Both *P. sojae* and *S. sclerotiorum* showed linear behavior with respect to spore germination (Table 9). The reduction of the germination of *P. sojae* spores in relation to control was 22% and 32% at concentrations of 15% and 20%. In contrast, at concentration of 10%, the extract showed 25% reduction in the germination of *S. sclerotiorum* spores and at concentrations of 15% and 20%, reductions were even greater, 28% and 44%, respectively.

Table 8. Unfolding of the fungal species interaction within each concentration level of the extract of *C. procera*. Variable answer: Germination

Fungi	Concentrations of the aqueous extract of <i>C. procera</i>				
	0%	5%	10%	15%	20%
<i>P. sojae</i>	100a	100a	98.50b	78.00a	68.00b
<i>S. sclerotiorum</i>	100a	94.5a	75.75a	71.75a	56.00a
Minimal significant difference	7.04				

Note. Means followed by the same letter in the column do not differ by the F test at 5% probability.

Table 9. Unfolding of the interaction concentrations of *C. procera* extract within each level of procera fungus species. Variable answer: Germination

Fungi	Equation	R ²
<i>P. sojae</i>	$y = 106.10* - 1.72*x$	82.17%
<i>S. sclerotiorum</i>	$y = 101.75* - 2.21*x$	96.46%

Note. * Significant at 5% probability by the t test.

4. Discussion

Results show that at lower concentrations, extracts affected more *P. sojae* than *S. sclerotiorum* in relation to mycelial growth rate. This variable is a distinctive quality that demonstrates the variation in sensitivity from one pathogen to another when under conditions unfavorable to its development (Sittisart et al., 2017; Fourie et al., 2019). When the growth rate is lower, it means that the fungus was more affected by the presence of the extract in the culture medium (Sittisart et al., 2017). The effectiveness of the antifungal action on the *in vitro* development of phytopathogens shows that compounds present in *C. procera* are potential biofungicides (Khazada et al., 2016).

The diameter of the phytopathogen colony was negatively affected by the application of the aqueous extract. This is consistent with research that showed the efficiency of *C. procera* extract on soil phytopathogens (Etaware, 2019; Ali et al., 2020). Numerous fungal disease management alternatives have been implemented to manage plant pathogenic fungi (Carmona-Hernandez et al., 2019; O'Brien, 2017; Varo et al., 2017). Agrochemicals provide rapid effects on phytopathogens but cause risks to human health and environment (Jabeen et al., 2013; Bello et al., 2020; Etaware, 2019). In our study, *C. procera* has shown significant effect on *P. sojae* and *S. sclerotiorum* even at low concentrations.

Sporulation and germination were also affected by the aqueous extract. This antifungal activity is due to different types of secondary metabolites, such as glycosides, alkaloids and calotropin (Morsy et al., 2016). *C. procera* has a type of protein in its composition that facilitates the permeabilization of the membrane of spores and hyphae of fungi, facilitating its deterioration (Ranjit et al., 2012). *C. procera* inhibited the germination of *Fusarium solani*, *Neurospora* sp. and *Colletotrichum gloeosporioides* (Freitas et al., 2011). Our study demonstrated that *C. procera* can be used for the alternative management of disease-causing fungi in soybeans. Thus, this method can contribute to minimize the risk and danger of toxic fungicides. Future studies with this extract will identify the active compounds responsible for its fungicidal activity.

5. Conclusions

C. procera aqueous extract has fungicidal activity, reducing the mycelial growth rate, colony diameter, sporulation and germination of *P. sojae* and *S. Sclerotiorum in vitro*.

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