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Antifungal activity of *Cenostigma pluviosum* var. *peltophoroides* extract against *Colletotrichum* isolates associated with soybean anthracnose: effects on sporulation, conidial germination, and appressorium formation

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Abstract. Plant-derived compounds have been widely investigated as potential alternatives to synthetic fungicides in plant disease management. This study evaluated the antifungal activity of an ethanolic extract obtained from leaves of *Cenostigma pluviosum* var. *peltophoroides* against *Colletotrichum* spp. isolated from soybean plants affected by anthracnose. The isolates were obtained from soybean fields located in three municipalities in the state of Mato Grosso, Brazil. Antifungal activity was evaluated using in vitro assays, including disk-diffusion and spreading methods, to assess mycelial growth inhibition, mycelial growth rate index, sporulation inhibition, conidial germination, and appressorium formation. The extract showed limited inhibition of mycelial growth but significantly reduced sporulation and strongly inhibited appressorium formation, reaching up to 92.30% inhibition. The spreading method showed greater sensitivity for detecting sporulation inhibition compared with the disk-diffusion method. These results indicate that the extract interferes with important stages of the fungal infection process and may represent a potential source of bioactive compounds for alternative management strategies for soybean anthracnose. Further studies are required to identify the active compounds and evaluate their effectiveness under greenhouse and field conditions.
Keywords: Infection structures,botanical extracts,antifungal compounds, plant secondary metabolites, sustainable disease management.

Introduction

Soybean anthracnose is widespread in Brazilian soybean-producing regions and can be caused by several species of the genus *Colletotrichum* (Moraes et al., 2020). In the Brazilian state of Mato Grosso, at least five *Colletotrichum* species have been reported in association with soybean anthracnose, including *C. truncatum* (Rogério et al., 2017), *C. sojae* (Barbieri et al., 2017; Damm, 2019; Castro, 2018), *C. musicola* (Castro, 2018), *C. plurivorum* (Damm, 2019), and *C. chlorophyti* (Takaki Ino et al., 2024). Soybean anthracnose is considered one of the major limiting factors in soybean production and may cause severe epidemics and substantial yield losses under favorable environmental conditions (Boufleur et al., 2021).

Anthracnose can affect soybean plants at all developmental stages and may occur in multiple plant organs. Symptoms are typically characterized by dark, irregular, and depressed lesions that may occur during pre- and post-emergence damping-off as well as on leaves, stems, petioles, and pods. In severe cases, infection can lead to premature defoliation of soybean plants (Yang et al., 2014).

Disease development is favored by warm and humid environmental conditions. However, information on the life cycle and epidemiology of several *Colletotrichum* species associated with soybean anthracnose remains limited. Among these species, *C. truncatum* is the most extensively studied and has been reported to survive on seeds, crop residues, and weeds. This pathogen can also form microsclerotia, which contribute to its persistence and survival in the field (Hartman et al., 1986; Khan; Sinclair, 1991; Yang; Hartman, 2016; Tikami et al., 2023). The limited understanding of the biology and epidemiology of *Colletotrichum* species associated with soybean anthracnose may hinder the development of effective disease management strategies.

In addition, several studies have reported reduced efficacy of chemical control strategies against soybean anthracnose (Dias et al., 2016; Santos et al., 2023; Silva, 2018; Rogério et al., 2024; Mello et al., 2024). These limitations highlight the need to explore alternative or complementary approaches for disease management.

In this context, plant-derived products have received increasing attention as potential alternatives or complements to synthetic fungicides in plant disease management. Botanical extracts contain a wide range of secondary metabolites, including phenolics, flavonoids, terpenoids, and alkaloids, many of which exhibit antimicrobial and antifungal activity against plant pathogens (Tripathi & Dubey, 2004; Pusztahelyi et al., 2015). Several studies have shown that plant extracts can inhibit important developmental stages of phytopathogenic fungi, including mycelial growth, sporulation, and conidial germination in species of *Colletotrichum* (Cowan, 1999; Tripathi&Dubey, 2004). In addition to their antifungal properties, plant-derived products are often considered environmentally compatible and may reduce the selection pressure associated with intensive fungicide use in agricultural systems (Dayan et al., 2009). However, the biological activity of botanical extracts may vary depending on the plant species, plant tissue, extraction method, and pathogen isolate, requiring experimental validation for each host–pathogen system (Cowan, 1999).

Among potential sources of bioactive compounds, *Cenostigma pluviosum* var. *peltophoroides* (syn. *Poincianella pluviosa* var. *peltophoroides*) is a tree species native to Brazil that has been traditionally used for medicinal purposes and has been reported to contain biologically active secondary metabolites, suggesting potential antimicrobial properties (Lorenzi, 2008; Domingos et al., 2019; Souza et al., 2018).

Despite the growing interest in plant-derived antifungal compounds, information remains limited regarding the activity of extracts from *C. pluviosum* var. *peltophoroides* against *Colletotrichum* species associated with soybean anthracnose. Although several studies have investigated botanical extracts against phytopathogenic fungi, research evaluating their effects on multiple stages of the fungal infection process, including mycelial growth, sporulation, conidial germination, and appressorium formation, remains scarce for *Colletotrichum* species associated with soybean.

Understanding how plant-derived compounds affect these key stages of fungal development may contribute to the identification of alternative strategies for disease management.

Therefore, the objective of this study was to evaluate the antifungal activity of the ethanolic extract obtained from leaves of *C. pluviosum* var. *peltophoroides* against *Colletotrichum* spp. isolated from soybean, using different in vitro assays to assess its effects on fungal growth and reproductive structures.

Material and Methods

Plant material and extract preparation

Experiments were conducted at the Plant Pathology Laboratory of the Federal University of Mato Grosso, Sinop Campus (UFMT–Sinop), Brazil. These experiments were carried out as part of a broader research project on the bioactivity of plant-derived compounds against phytopathogenic fungi.

Leaves of *Cenostigma pluviosum* var. *peltophoroides*were collected in the municipality of Sinop, Mato Grosso, Brazil. A voucher specimen (CNMT 6065) was deposited in the Centro-Norte-Mato-Grosso Herbarium at UFMT–Sinop.

Leaves were dried at room temperature and ground into powder. The plant material was extracted by maceration using 95% ethanol. Four successive macerations were performed. The resulting solutions were filtered, pooled, and concentrated using a rotary evaporator at 40°C to obtain the crude ethanolic extract.

A stock solution of the extract was prepared by dissolving the crude ethanolic extract in sterile ultrapure water at a concentration of 0.8 mg mL^{-1} and subsequently filtered through a 0.45 µm membrane. Working solutions were prepared from this stock solution at concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 mg mL^{-1}.

Fungal isolates

Three fungal isolates were used in this study: LU-2 (*Colletotrichum truncatum*) and IT-4 and SI-9 (*Colletotrichum* sp.). These isolates were obtained from soybean fields located in the municipalities of Lucas do Rio Verde, Itaúba, and Sinop in the state of Mato Grosso, Brazil. The isolates were obtained from the mycological collection of the Federal University of Mato Grosso, Sinop Campus. Species identification of isolate LU-2 was previously confirmed based on morphological characteristics consistent with *C. truncatum*. Isolates IT-4 and SI-9 were maintained as *Colletotrichum* sp. because species-level identification based on molecular markers was not available at the time the experiment was conducted.

Mycelial growth and sporulation assays

The effect of the plant extract on mycelial growth and sporulation was evaluated using five concentrations (0.1, 0.2, 0.3, 0.4, and 0.5 mg mL^{-1}) and two application methods.

In the first method, sterile filter paper discs (5 mm diameter) were soaked in the extract solutions and placed onto Petri dishes containing Potato Dextrose Agar (PDA; Acumedia®) prepared at 39 g L^{-1}. The discs were gently pressed onto the agar surface to ensure full contact with the culture medium.

In the second method, 100 µL of each extract concentration was spread over the surface of PDA plates. The plates were allowed to rest for 1 h before inoculation.

Fungal inoculation was performed by placing a mycelial plug from an actively growing

colony of *Colletotrichum* spp. in the center of each plate.

Sterile distilled water was used as the negative control, and the fungicide mixture trifloxystrobin + prothioconazole (300 mL ha^{-1}) was used as the positive control.

Five replicates were used for each treatment. Plates were incubated at 26 ± 2 °C under a 12-h photoperiod. Colony diameter was measured daily from 24 h after inoculation onward using two perpendicular measurements, and evaluations continued until colonies in at least one treatment reached two-thirds of the plate surface.

Mycelial growth rate, mycelial growth rate index, and sporulation were determined according to Oliveira (1991).

Sporulation assessment

The effect of the extract on conidial production was evaluated after mycelial growth had ceased. Ten milliliters of sterile distilled water were added to the surface of each plate, and the colony was gently scraped to release conidia. The resulting suspension was filtered through gauze. One mL aliquot of the suspension was used for conidial counting in a Neubauer hemocytometer, following the method adapted from Balbi-Peña et al. (2006).

Conidial germination and appressorium formation

The antifungal activity of the extract on conidial germination and appressorium formation was evaluated according to the methodology described by Bonaldo et al. (2004).

Aliquots of 40 µL of conidial suspension (1 × 10^4 conidia mL^{-1}) were mixed with 40 µL of each treatment concentration. The fungicide fluxapyroxad + pyraclostrobin (100 mL ha^{-1}) was used as the positive control. Each mixture was transferred to wells of a sterile, flat-bottom 96-well microtiter plate without a lid, with eight replicates per treatment. Plates were incubated in a BOD incubator (Caltech®) at 25 ± 2 °C in the dark for 20 h.

After incubation, germination was stopped by adding 20 µL of lactophenol blue. Conidia were examined under a light microscope at 400× magnification. One hundred conidia were counted per well, and conidia were considered germinated when the germ tube was equal to or longer than the width of the conidium.

Statistical analysis

The experiments were arranged in a completely randomized design (CRD). Treatments consisted of five concentrations of the ethanolic extract (0.1, 0.2, 0.3, 0.4, and 0.5 mg mL^{-1}), a negative control consisting of sterile distilled water, and a positive control consisting of a commercial fungicide.

For mycelial growth and sporulation assays, five replicates were used for each treatment. For conidial germination and appressorium formation assays, eight replicates were used for each treatment.

Experiments were analyzed separately for each fungal isolate and assay. The response variables evaluated included mycelial growth inhibition, mycelial growth rate index, sporulation inhibition, conidial germination inhibition, and appressorium formation inhibition. Each variable was analyzed independently. Data were subjected to analysis of variance (ANOVA), and when the F test was significant ($P < 0.05$), treatment means were compared using the Scott–Knott test at the 5% probability level. Statistical analyses were performed using SISVAR software (Ferreira, 2011).

Results and discussion

Effect of the extract on mycelial growth

The ethanolic extract of *Cenostigma pluviosum* var. *peltophoroides* showed limited inhibition on the mycelial growth of the evaluated *Colletotrichum* isolates. Significant differences ($P < 0.05$) were observed only for the SI-9 isolate when both disk-diffusion and spreading methodologies were evaluated. However, the commercial fungicide treatment was the only treatment that effectively inhibited mycelial growth in this isolate (Table 1).

Table 1. Mycelial growth inhibition (%) of *Colletotrichum* isolates treated with different concentrations of *C. pluviosum* var. *peltophoroides* extract using disk-diffusion and spreading methods.

Treatments	Disk-diffusion			Spreading		
	IT-4 - (*Colletotrichum* sp.)^{ns}	LU-2 - (*C. truncatum*) ^{ns}	SI-9 - (*Colletotrichum* sp.)*	IT-4 - (*Colletotrichum* sp.)^{ns}	LU-2 - (*C. truncatum*)^{ns}	SI-9 - (*Colletotrichum* sp.)*
0.1 mg.mL^{-1}	2.16	3.66	23.30 a	15.07	6.82	3.02 a
0.2 mg mL^{-1}	1.78	1.80	20.01 a	13.51	3.55	0.94 a
0.3 mg mL^{-1}	0.00	5.54	18.50 a	4.89	5.40	5.08 a
0.4 mg mL^{-1}	0.82	0.00	18.60 a	17.01	4.72	2.22 a
0.5 mg mL^{-1}	0.66	1.14	27.85 a	14.90	10.02	0.86 a
Fungicide	0.50	1.99	45.37 b	20.34	11.93	15.69 b

Means followed by the same letter within each column do not differ according to the Scott–Knott test ($P < 0.05$).
*: significant at $P < 0.05$; ^{ns}: not significant.

Overall, the extract exhibited a low inhibitory effect on the vegetative growth of the tested isolates. Similar results were observed for both disk-diffusion and spreading methods, suggesting that the method of application did not substantially influence the antifungal activity of the extract under the evaluated conditions.

Mycelial growth rate

In the mycelial growth rate index (MGRI) obtained using the disk-diffusion method, significant differences were observed for isolates LU-2 and SI-9 ($P < 0.05$). For the LU-2 isolate (*C. truncatum*), concentrations of 0.1 and 0.2 mg mL^{-1} produced results similar to the fungicide treatment (Table 2).

Using the spreading method, significant differences were observed for isolates IT-4 and SI-9 ($P < 0.05$). For isolate IT-4, all tested concentrations of the extract, except 0.3 mg mL^{-1}, showed results statistically similar to the fungicide treatment (Table 2). These results indicate that the ethanolic extract had a relatively weak effect on mycelial growth dynamics of *Colletotrichum* spp., suggesting that the antifungal activity of the extract may be more strongly associated with other stages of the fungal life cycle rather than with vegetative growth.

Table 2. Mycelial growth rate index of *Colletotrichum* isolates treated with different concentrations of *C. pluviosum* var. *peltophoroides* extract using disk-diffusion and spreading methods.

Treatments	Disk-diffusion			Spreading		
	IT-4 - (*Colletotrichum* sp.)^{ns}	LU-2 - (*C. truncatum*)*	SI-9 - (*Colletotrichum* sp.)*	IT-4 - (*Colletotrichum* sp.) - *	LU-2 - (*C. truncatum*) ^{ns}	SI-9 - (*Colletotrichum* sp.)*
0.1 mg mL^{-1}	0.34	0.65 b	0.51 b	0.45 b	0.72	0.72 a
0.2 mg mL^{-1}	0.36	0.61 b	0.53 b	0.45 b	0.73	0.76 a
0.3 mg mL^{-1}	0.36	0.69 a	0.54 b	0.51 a	0.75	0.71 a
0.4 mg mL^{-1}	0.36	0.73 a	0.56 b	0.45 b	0.73	0.77 a
0.5 mg mL^{-1}	0.35	0.69 a	0.48 b	0.46 b	0.70	0.80 a
Distilled water	0.35	0.65 b	0.67 a	0.54 a	0.76	0.72 a
Fungicide	0.35	0.64 b	0.36 c	0.43 b	0.68	0.61 b

Means followed by the same letter within each column do not differ according to the Scott–Knott test ($P < 0.05$).
*: significant at $P < 0.05$; ^{ns}: not significant.

Effect on sporulation

Significant inhibition of sporulation ($P < 0.05$) was observed for the LU-2 isolate (*C. truncatum*) when the spreading method was used. Under these conditions, concentrations of 0.1, 0.2, 0.3, and 0.5 mg mL^{-1} did not differ statistically from the fungicide treatment (Table 3).

These results suggest that the extract may interfere with fungal reproductive processes. The spreading method appearedmore suitable for evaluating the effect of the extract on sporulation, likely due to improved contact between the extract and the fungal colony on the culture medium surface.

Table 3. Sporulation inhibition (%) of *Colletotrichum* isolates treated with different concentrations of *C. pluviosum* var. *peltophoroides* extract using disk-diffusion and spreading methods.

Treatments	Disk-diffusion			Spreading		
	IT-4 - (*Colletotrichum* sp.)^{ns}	LU-2 - (*C. truncatum*) ^{ns}	SI-9 - (*Colletotrichum* sp.)^{ns}	IT-4 - (*Colletotrichum* sp.)^{ns}	LU-2 - (*C. truncatum*)*	SI-9 - (*Colletotrichum* sp.)^{ns}
0.1 mg mL^{-1}	0.00	20.91	0.00	45.18	62.59 b	80.00
0.2 mg mL^{-1}	15.55	7.69	20.00	34.02	70.89 b	60.00
0.3 mg mL^{-1}	14.72	27.51	0.00	46.28	40.00 b	40.00
0.4 mg mL^{-1}	8.88	38.96	20.00	40.15	0.00 a	20.00
0.5 mg mL^{-1}	0.00	39.04	40.00	29.51	61.15 b	80.00
Fungicide	17.44	39.78	40.00	61.84	95.35 b	60.00

Means followed by the same letter within each column do not differ according to the Scott–Knott test ($P < 0.05$).
*: significant at $P < 0.05$; ^{ns}: not significant.

Effect on conidial germination and appressorium formation

Significant inhibition of conidial germination was observed for isolates IT-4 and SI-9 ($P < 0.05$). For the IT-4 isolate, the concentration of 0.5 mg mL^{-1} produced inhibition levels comparable to those obtained with the fungicide treatment. For the SI-9 isolate, the same concentration showed even greater inhibition than the commercial fungicide used as the positive control (Table 4).

Appressorium formation was significantly inhibited in all evaluated isolates ($P < 0.05$). The concentration of 0.5 mg mL^{-1} significantly reduced appressorium formation by 54.39%, 92.30%, and 92.30% for LU-2 (*C. truncatum*), IT-4, and SI-9 isolates, respectively (Table 4).

The inhibition of appressorium formation is particularly relevant from a phytopathological perspective because this structure is essential for host penetration and successful infection by *Colletotrichum* species. Therefore, compounds capable of interfering with this stage of fungal development may contribute to alternative disease management strategies.

Table 4. Inhibition of conidial germination and appressorium formationof *Colletotrichum* isolates treated with different concentrations of *C. pluviosum* var. *peltophoroides* extract.

Treatments	Inhibitionofconidialgermination (%)			Inhibition appressorium formation (%)		
	IT-4 - (*Colletotrichum* sp.)*	LU-2 - (*C. truncatum*)*	SI-9 - (*Colletotrichum* sp.)*	IT-4 - (*Colletotrichum* sp.)*	LU-2 - (*C. truncatum*)*	SI-9 - (*Colletotrichum* sp.)*
0.1 mg mL^{-1}	5.50 a	0.88 a	8.43 a	14.02 a	1.76 a	12.35 a
0.2 mg mL^{-1}	11.91 a	1.00 a	7.45 a	12.27 a	1.25 a	12.27 a
0.3 mg mL^{-1}	2.23 a	1.87 a	11.83 a	7.07 a	2.75 a	7.07 a
0.4 mg mL^{-1}	0.00 a	2.01 a	12.19 a	5.46 a	3.65 a	5.46 a
0.5 mg mL^{-1}	34.09 b	14.77 b	21.06 b	92.30 b	54.39 b	92.30 b
Fungicide	24.18 b	1.63 a	5.96 a	25.76 a	1.75 a	25.76 a

Averages in columns followed by the same letter do not differ by the Scott-Knott test at 5% probability. *: Significant at 5%.

Previous phytochemical analyses of *C. pluviosum* var. *peltophoroides* have identified the presence of several classes of secondary metabolites, including condensed tannins, phenolic compounds, steroids, terpenoids, flavonoids, saponins, and alkaloids (Cunha et al., 2014). Many of these compounds are widely reported to possess antimicrobial activity and may contribute to the inhibitory effects observed in this study.

Phenolic compounds and terpenoids, which are commonly found in species of the Fabaceae family, are known to interfere with fungal cell structures and metabolic processes. In addition, biflavonoid compounds have been previously identified in *C. pluviosum var. peltophoroides*, representing relatively rare chemical structures within the genus (Bahia et al., 2010; Zanin et al., 2012).

Several studies have investigated the antimicrobial activity of plant extracts from Fabaceae species. Pereira et al. (2006) reported antimicrobial activity of ethanolic extracts of *Poincianella pyramidalis* against several microorganisms, while Cavalheiro et al. (2009) evaluated antifungal activity of plant extracts against different phytopathogenic fungi. However, these studies did not evaluate the activity of *C. pluviosum var. peltophoroides*against *Colletotrichum* species associated with soybean anthracnose.

The results obtained in this study demonstrate that the ethanolic extract of *C. pluviosum* var. *peltophoroides* can interfere with important stages of the fungal life cycle, particularly sporulation, conidial germination, and appressorium formation. Although the extract showed limited effects on mycelial growth, its strong inhibitory activity on infection-related structures highlights its potential as a source of antifungal compounds.

Recent studies have also supported the antifungal potential of plant-derived compounds against *Colletotrichum* species associated with anthracnose. Botanical extracts were able to strongly inhibit fungal growth and spore germination of *Colletotrichum boninense*, in addition to reducing anthracnose under protected cultivation conditions (Kushaha et al., 2024).

Similarly, Christopher et al. (2023) demonstrated antifungal activity of different extracts of *Leonotisnepetifolia* against *Colletotrichum* species causing bean anthracnose, while Sudirga et al. (2023) reported that leaf extracts from selected plant species inhibited the growth of *C. acutatum* associated with chili anthracnose. Together, these studies reinforce the potential of plant-derived compounds as alternative tools for the management of diseases caused by *Colletotrichum* spp.

Plant extracts frequently require relatively high concentrations, compared with synthetic fungicides, to produce measurable antifungal effects. For example, Bonaldo et al. (2007) reported inhibition of several phytopathogenic fungi only when crude plant extracts were applied at concentrations of 20%. Despite this limitation, plant-derived compounds remain promising sources of bioactive molecules with potential use in plant disease management.

However, in this study, inhibitory effects were observed even at relatively low concentrations of the ethanolic extract (0.1–0.5 mg mL^{-1}), particularly in processes related to fungal reproduction and infection. This suggests that compounds present in *C. pluviosum var. peltophoroides* may interfere with key physiological processes of the pathogen rather than directly inhibiting vegetative growth.

Conclusion

The ethanolic extract of *Cenostigma pluviosum* var. *peltophoroides* showed antifungal activity against *Colletotrichum* spp. isolated from soybean. Although the extract exhibited limited inhibition of mycelial growth, it significantly affected important stages of the fungal life cycle, particularly sporulation, conidial germination, and appressorium formation.

The strong inhibition of infection-related structures suggests that compounds present in this plant may interfere with key processes involved in pathogen establishment.

These findings highlight the potential of *C. pluviosum* var. *peltophoroides* as a source of bioactive compounds for the development of alternative strategies for soybean anthracnose management.

Further studies are needed to identify the active compounds responsible for the antifungal activity and to evaluate their effectiveness under greenhouse and field conditions.

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