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Evaluation of the Fungitoxic Activity of Copaiba Oil (Copaifera spp.) from Western Amazon against Anthracnose (Colletotrichum gloeosporioides (Penz.) Penz. & Sacc.) in Papaya (Carica papaya L.)

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ABSTRACT

The genus Copaifera is not only relevant to the timber industry but also stands out for its resin oil, a transparent, viscous, and fragrant exudate. This product, commonly used in folk medicine, symbolizes the wealth of forest-derived products and their appreciation. In line with the Sustainable Development Goals (SDGs) metrics, it is essential that its exploitation is linked to sustainability, promoting regional development and emphasizing the importance of protecting and valuing the region's natural resources. Therefore, the objective of this study was to evaluate the physicochemical characteristics of Copaiba resin oil varieties collected at farmers' markets and assess its fungicidal potential in controlling Colletotrichum gloeosporioides, the causative agent of papaya anthracnose (Carica papaya). The fungus Colletotrichum gloeosporioides was isolated from diseased papaya fruits. The following treatments were used: a control and doses of 50, 100, 150, and 200 µL/mL of Copaiba oil, with each dose incorporated into 20 mL of BDA (Potato, Dextrose, and Agar) culture medium, to determine the effect of the oil on the mycelial growth of the phytopathogen. The data were subjected to analysis of variance (ANOVA) and regression analysis. When evaluating the effect of oil varieties, it was found that the greatest inhibitory effect was observed for variety 2, with the highest inhibition power caused by doses of 100 and 200 µL/mL in variety 2.

Keywords: Resin, Inhibitory effect, Alternative control, Bioprospecting, Sustainability.

1 INTRODUCTION

Copaifera spp. are trees native to the tropical regions of Latin America and West Africa (Paula-Souza, et al., 2022; Diefenbach, et al., 2017). In Brazil, there are more than twenty species of Copaifera, the most abundant being C. officinalis (Jacq.) L., C. guianensis Desf., C. reticulata Ducke,

C. multijuga Hayne, C. confertiflora Benth., C. langsdorffii Desf., C. coriacea Mart. and C. cearensis Huber ex Ducke (Rigamonte-Azevedo, et al., 2004), easily sighted throughout the territory of the Amazon region (Paula-Souza, et al., 2022; Pierre, et al., 2009). The genus Copaifera is widely used in the timber industry, but its emphasis is on the use of resin oil, a transparent, viscous exudate with a strong odor, which can turn into resin when exposed to air and light (Mendonça & Onofre, 2009).

Popularly, resin oil is known as copaiba oil, whose antibacterial properties (Bardají, et al., 2016; Ziech, et al., 2013; Santos, et al., 2008; Pacheco, et al., 2006) and antifungals (De Brito & Moreira, 2017; Abreu, et al., 2014) are widely described in the literature. Its chemical properties are also associated with the treatment of hemolytic lesions and activities (Fernandes, et al., 2023).

The microbiostatic action of Copaiba essential oil (Copaifera spp.) is already widely known and explored in the control of plant diseases (Amazon Oil, 2023; Veloso, et al., 2020; De Souza Oliveira, et al., 2018; Gomes, et al., 2016). Silva (2019) evaluated the antifungal potential of five essential oils (cloves, sesame, sunflower, copaiba and fennel) in the control of Fusarium sp. and observed that copaiba essential oil presented the second best result for the mycelial growth velocity index (IVCM), average colony diameter (DMC) and percentage of mycelial growth inhibition (PIC).

The inhibitory effect of copaiba oil was also reported by Nóbrega (2018) against the phytopathogens Alternaria alternata and Colletotrichum musae, and by Araújo, Toledo & Soares (2018), where copaiba oil showed a reduction in the mycelial growth of Colletotrichum spp. at all concentrations when compared to the other treatments. Similar results were observed by Porcino (2018), where copaiba essential oil was highly efficient in inhibiting the mycelial growth of the pathogen Alternaria alternata f. sp. Citri, causal agent of Alternaria brown spot on tangerines.

The active ingredient of resin oil is the result of a solution of diterpene acids, being an essential oil consisting of sesquiterpenes. The main sesquiterpenes include β-caryophyllene, with anti-inflammatory and antifungal action, and β-bisabolene, which acts as an analgesic and also has anti-inflammatory properties (Fernandes, et al., 2023; Yamaguchi & Garcia, 2012; Veiga Júnior & Pinto, 2002).

Al-Reza et al. (2010) state that plant essential oils can be used as alternative strategies to control pathogenic plant fungi because they are a virtually rich source of bioactive substances that can lead to the development of new classes of disease control agents. However, it is difficult to compare results obtained in different studies, as several factors can alter the composition and quantity of oils, depending on the geographic region, variety, age of the plant, drying method, and oil extraction method, among others.

Similarly, Fernandes et al. (2023) report that Copaifera has chemical and pharmacological properties favorable to the treatment of lesions and wounds, such as the control of inflammatory pain, reduction of inflammatory reaction, re-epithelialization and tissue repair, angiogenesis, wound

retraction, and scar remodeling. The presence of bioactive compounds, such as diterpenes, 3-hydroxy-copalic, sesquiterpenes, and kolavic-15-methyl ester, is also highlighted.

The extraction and commercialization of copaiba oil generate a dynamic of economic activities that take advantage of the potential of the Amazon rainforest without destroying it, generating income and maintaining local communities. The pharmacological and commercial potential of copaiba oil, especially for the Amazon region, still justifies studies aimed at the technological development of products containing this raw material (De Lima & Andrade, 2010; Lira & Chaves, 2015).

Within this context, attention is drawn to the possible use of copaiba oil in the control of the fungus Colletotrichum gloeosporioides, the main pathogen that attacks papaya fruits, causing the disease known as anthracnose (Dantas, et al., 2013). The chemical control of anthracnose is not fully effective, as it increases the cost of production and generates negative impacts on the environment and human health (Machado, 2013). The use of vegetable oils, such as copaiba, has aroused great interest due to the presence of bioactive substances that can be used in the management of plant diseases. Thus, it is important to carry out studies that enable the use of copaiba oil for tests of antifungal activity in the control of papaya anthracnose, since the oil is found naturally and can be used as a sustainable and economically viable means (Veiga Júnior and Pinto, 2002).

Thus, the objective of this study was to evaluate the physicochemical characteristics of copaiba oil-resin varieties collected in farmers' markets and to evaluate the fungicidal potential in the control of *Colletotrichum gloeosporioides,* the causative agent of papaya anthracnose (*Carica papaya*).

2 METHODOLOGY

The work was carried out at the Laboratory of Chemistry and Soils and Phytopathology of the Federal University of Acre, Campus Floresta Centro Multidisciplinar – CMULTI, in Cruzeiro do Sul, Acre – Brazil. The predominant climate of the region, according to the Köppen classification, is type Af, characterized as hot and humid tropical, with an average annual temperature of 24°C (Alvares, et al., 2014).

2.1 OBTAINING THE VARIETIES OF *COPAIFERA* SP.

Varieties of *Copaifera* sp. oils were randomly acquired at a family farmers' fair in Ariquemes-Rondônia in partnership with the Reca (2023) project (Dense Consortium Economic Reforestation) and named copaiba 1, 2, 3 and 4. The varieties were stored in a closed amber flask and preserved at a temperature of -5 ± 1oC to avoid oxidation and degradation by light radiation until the moment of analysis.

2.2 VISCOSITY DETERMINATION

The viscosity of the oil-resins was determined using a rotary axis viscometer (Quimis). 50 mL of oil-resin was measured in the sample holder and kept at a temperature of 20 ± 1oC for 10 min. After obtaining a constant temperature, the resin oils were subjected to rotation at 30 rpm for 10 min and viscosity was measured. The viscosity was performed in triplicate, with the mean density value and its respective standard deviation (σ) Lutz (2008).

2.3 DENSITY DETERMINATION

The density of the liquid was determined by the difference in the mass of the liquid by the volume occupied. The amount of resin oils was 50 mL using a pycnometer at a temperature of 20 ± 1oC. The density of the oil-resins was performed in triplicate, with the mean density value and its respective standard deviation (σ), Lutz, (2008).

2.4 DETERMINATION OF THE REFRACTIVE INDEX

The ABBE benchtop refractometer (Quimis) was used to determine the refractive index of the oil-resins. Approximately 4 drops of oil-resins were placed in the refractometer sample holder and after reaching 20 ± 1oC, the refractive index for each variety was evaluated. It was performed in triplicate, with its respective standard deviation (σ), Lutz, (2008).

2.5 DETERMINATION OF ACIDITY

2 g of the oil-resins were weighed in a 125 mL Erlenmeyer flask and 25 mL were added, previously prepared with ether-alcohol (2:1). Then, two drops of phenolphthalein indicator were added. It was titrated with 0.1 M sodium hydroxide solution, until the appearance of pink color, and left for 30 seconds, after this time, the amount of NaOH spent per gram of oil was calculated according to the Lutz (2008) standards.

2.6 EVALUATION OF THE FUNGICIDAL ACTIVITY OF COPAIBA OIL-RESIN

The fungus *Colletotrichum gloeosporioides* was isolated from papaya fruits (*Carica papaya* L.) who showed symptoms of anthracnose disease. The infected fruits were obtained in the city of Cruzeiro do Sul, Acre. The fungal isolation methodology described by Menezes and Silva-Hanlin (1997) was used. With a flambéed scalpel, a cut was made in the affected area and the incision was pressed to facilitate the opening of the cut in the fruit; Then, with the aid of a stylet, the fragments were removed from the transition region of the lesion (the area between healthy and diseased tissue). Then, the fragments were immersed in 70% alcohol for one min, in 1.5% sodium hypochlorite solution for 30 seconds, and then washed with sterile and distilled water. After these procedures, plating was

performed in 20 mL in the PDA culture medium (Potato Dextrose and Agar), four tissue fragments were placed per plate, which were distributed equidistantly. The cultures were incubated at room temperature of 25ºC under fluorescent light.

2.7 EVALUATION OF THE ANTIFUNGAL ACTIVITY OF COPAIBA OIL

In a laminar flow hood, increasing doses of copaiba oil (0, 50, 100, 150 and 200 µL/mL) were added to the still melting PDA culture medium and poured into sterile 9 cm Petri dishes. Then, a disk containing the mycelium of the fungus measuring 5 mm in diameter was deposited in the center of each plate. The same procedure was performed with the control sample. The plates were sealed with cling paper, identified and incubated in BOD under a 12-hour photoperiod at a temperature of 25 °C.

The mycelial growth evaluations were carried out by means of daily observations initiated 24 hours after the installation of the experiment in an orthogonal position and lasted until the moment when the first fungal colony of the treatment reached its maximum development on the surface of the Petri dish culture medium. To calculate the mycelial growth index (MCI) or mycelial growth rate (MCT), the formula described by Salgado et al. (2003) was used, which states that the variables contained in the equation for the calculation of the mycelial growth index (MCI) are the evaluations of mycelial growth from the first to the last day (C1, C2, C3, C4...,Cn) and the number of the day of the assessment (N1, N2, N3, N4...,Nn). Given by:

$$ICM = \frac{C_1}{N_1} + \frac{C_2}{N_2} + \frac{C_3}{N_3} + \frac{C_4}{N_4} \ldots \frac{C_n}{N_n}$$

To calculate the percentage of mycelial growth inhibition (ICP), the methodology proposed by Garcia et al. (2012) was used, where the control diameter (DT) and the treatment diameter (DTRAT) are the variables involved. Given by:

$$PIC = \frac{DT - DTRAT}{DT} \times 100$$

The trials were conducted in a completely randomized design, with a factorial scheme involving 4 (oil varieties) x 5 (oil doses + control test of the control), with 5 replicates adopted for each treatment. The data were submitted to analysis of variance (ANOVA) and regression analysis, whose difference between the means of the treatments was tested by Tukey's test at 5% of significance

performed with the aid of the RStudio Core Team software, version 3.6.3 and with a package *ExpDes.pt, car, stats* and *fBasics.*

3 RESULTS AND DISCUSSION

3.1 PHYSICOCHEMICAL ANALYSIS OF OIL-RESIN

As shown in Table 1, variations were observed in the acidity index of the copaiba resin oils studied, with values ranging from 1.63 ± 0.02 mg of NaOH/g of oil for the copaiba 2 variety to 4.02 ± 0.02 for the copaiba 3 variety. These results are in accordance with the values established by Anvisa (2023), in which acidity index values close to 4.0 mg of NaOH/mL are considered acceptable for human consumption.

Regarding the viscosity analysis, it was found that copaiba oil 3 presented the highest viscosity value, 11.3 ± 0.4, while the sample representing copaiba 1 revealed the lowest value, 5 ± 0.6.

Table 1: Results of the analysis of acidity index, density, viscosity and refractive index of copaiba oil.

	Copaiba 1	Copaiba 2	Copaiba 3	Copaiba 4
Acidity (mgNaOH/g)	2.21 ± 0.01	1.63 ± 0.02	4.02 ± 0.02	2.85 ± 0.02
Density (g/mL)	0.93 ± 0.01	0.92 ± 0.01	0.94 ± 0.02	0.93 ± 0.01
Viscosity (mPa.s)	10 ± 0.3	5 ± 0.6	11.3 ± 0.4	10 ± 0.2
Refractive Index	1,503 ± 0,001	1,502 ± 0,001	1,505 ± 0,001	1,507 ± 0,001

Source: Authors

As shown in Table 1, the density values of copaiba oils did not show significant differences. Copaiba 2 oil had the lowest density, with 0.92 ± 0.01, while copaiba 3 oil had the highest density, with 0.94 ± 0.02.

However, with regard to the refractive index, all varieties of copaiba showed small variations in values. Copaíba 1, with a refractive index of 1.503 ± 0.001, and Copaiba 4, with a refractive index of 1.507 ± 0.001, stand out. These subtle differences can be attributed to the unique characteristics of each type of oil, including the degree of saturation of the bonds, the oxidation process, and the heat treatment (Lutz, 2008).

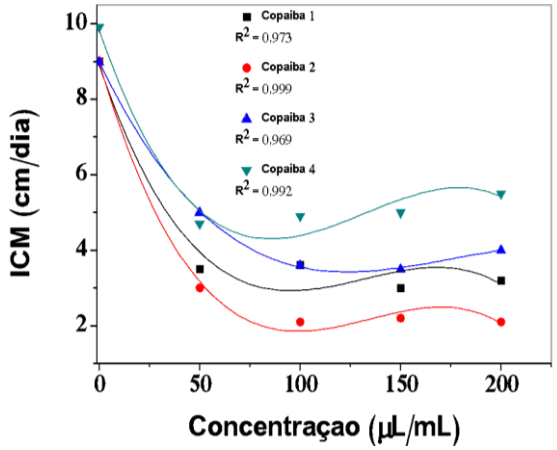
3.2 FUNGICIDAL ACTIVITY OF COPAIBA OIL

Regression analysis revealed statistically significant models indicating that copaiba oil varieties have an inhibitory effect on the mycelial growth of the pathogen. As shown in Figure 1, the mycelial growth curves of Colletotrichum gloesoiporioides for each variety and dose of copaiba oil show an inversely proportional correlation. Notably, the copaiba oil variety 2 showed a greater stimulus in the

inhibition of mycelial growth at the doses of 100 and 200 µL/mL, with a more significant inhibition at the dose of 100 µL. In contrast, the copaiba 4 variety exhibited the lowest inhibition of mycelial growth.

Similar results were obtained in previous studies by Sousa et al. (2012), who investigated the antifungal activity of copaiba oil against Colletotrichum gloeosporioides at various concentrations, achieving satisfactory results. In addition, Ishida et al. (2008) examined the fungicidal effect of copaiba oils extracted from different parts of the plant on Fusarium spp., the pathogen responsible for root and foot rot in black pepper plants.

Figure 1 - Regression analysis for the effect of copaiba oil doses on the mycelial growth of *Colletotrichum gloesoiporioides*



Source: Authors

For the mycelial growth index in the concentration range of 0 to 200.0 µL/mL of copaiba oils, a significant reduction was observed between the varieties of copaiba oils and between their concentrations (Table 2).

Table 2: Mycelial growth index (MCI) of *Colletotrichum gloesoiporioides* for copaiba (*Copaífera* sp.) oil varieties

Oil	Dose (µL/mL)				
	0	50	100	150	200
Copaiba 1	7.88 aB	3.46 bB	3.14 bB	3.32 bB	2.87 bC
Copaiba 2	7.88 aB	3.09 bB	2.09 cC	2.24 cC	2.17 cD
Copaiba 3	8.78 aA	5,09 bA	4,56 bcA	3,48 cB	4,17 dB
Copaiba 4	8,91 aA	4,73 cA	4,62 cA	5,06 bcA	5,51 bA

a,b – For each oil variety, mean doses of oils followed by the same lowercase letter do not differ significantly from each other, according to Tukey's test (α = 0.05).
A,B - For each oil dose, the means of the oil varieties followed by the same capital letter did not differ significantly from each other, according to Tukey's test (α = 0.05).

Source: Authors

The results presented in Table 2 indicate that, among the varieties of copaiba oil, the Copaiba 2 variety showed a more pronounced inhibitory effect at concentrations of 100, 150 and 200 µL/mL, differing statistically from the other varieties. This result is in line with the findings of Araujo Neto et al. (2014), who also observed the efficacy of copaiba oil in the control of anthracnose (Colletotrichum gloeosporioides) in yellow passion fruit trees.

The research by Sousa et al. (2019) analyzed copaiba and andiroba oils, as well as their combinations, in the control of the fungus Sclerotium rolfsii in tomato plants, showing that copaiba oils have significant potential against the fungus, with pure copaiba oil demonstrating the highest efficiency.

Figueredo et al. (2021) evaluated the in vitro sensitivity of the fungus Colletotrichum gloeosporioides to essential oils, such as basil and cassia cinnamon, obtaining minimum inhibitory concentrations (MIC) as low as 5 µL/mL for 100% inhibition of mycelial growth.

Similarly, Tofiño-Rivera et al. (2020) demonstrated the efficacy of Cymbopogon citratus essential oil encapsulated in chitosan-agar against Colletotrichum gloeosporioides, achieving a control index of 100% with a concentration of 4 µL/mL.

In the study conducted by Xia et al. (2020), an essential oil was extracted from the leaves of Murraya microphylla through steam distillation. This oil was evaluated for its fungicidal capacity against the fungus Colletotrichum gloeosporioides. It was determined that the concentration required to obtain antifungal effect was 0.125 mg/mL.

It is observed that, while the research by Figueredo et al. (2021), Tofiño-Rivera et al. (2020) and Xia et al. (2020) investigated the Minimum Inhibitory Concentration (MIC) of the fungus Colletotrichum gloeosporioides against essential oils obtained by hydrodistillation from different sources, copaiba oil extracted from the stem presented different results in terms of MIC, highlighting the uniqueness of its antifungal properties.

Table 3 shows the results of the mycelial growth inhibition (ICP) percentage calculations. Of the copaiba samples tested, copaiba 2 showed the highest inhibitory efficacy, ranging from 59.85% at the lowest dosage (50 µL/mL) to 69.92% at the highest dosage (200 µL/mL). In contrast, the copaiba 4 sample exhibited the lowest inhibitory effect at all dosages examined.

Table 3 - Effect of copaiba oil doses on the reduction of ICP (percentage of mycelial growth inhibition).

Oil	Dose (µL/mL)			
	50	100	150	200
Copaiba 1	51.58 aAB	54.14 aB	50.22 aB	57.89 aB
Copaiba 2	59,85 bA	63.91 abA	69.93 aA	69.92 aA
Copaiba 3	49.55 cB	52.13 bcBC	67.53 aA	60.67 abAB
Copaiba 4	45.25 aB	43.93 BC	37.47 BC	36.15 BC

a,b – For each oil variety, mean doses of oils followed by the same lowercase letter do not differ significantly from each other, according to Tukey's test (α = 0.05).
A,B - For each dose of oil, the means of the varieties of oils followed by the same capital letter did not differ significantly from each other, according to Tukey's test (α = 0.05).
Source: Authors

The results presented differ from those found by Nascimento et al. (2014), who, when applying various concentrations of copaiba oil (25, 50, 75 and 100 µL/mL) on the fungus Fusarium solani f. sp. glycines, which causes red rot in soybean roots, identified an inversely proportional relationship

between the mycelial growth of the fungus and the doses applied. The highest dose tested, 100 µL/mL, resulted in the smallest colony diameter measured at 1.88 cm, which is equivalent to an inhibition of 57.84%. In contrast, in the present study, the data did not show an inverse proportion, notably in variety 4, where the most significant inhibitory effect was observed with the lowest concentration tested, 50 µL/mL.

4 CONCLUSION

With the results obtained in the present study, it was possible to observe that copaiba oil showed an inhibitory effect on the mycelial growth of *Colletotrichum gloesoiporioides*, which can be a viable and sustainable alternative for the producer. The doses of 100 and 200 µL/mL showed the greatest reduction in the MCI (Mycelial Growth Index), and the greatest inhibitory effect was observed for copaiba 2 oil, which presented the lowest acidity index in the physicochemical parameters. These results show that the action of each oil variety is dependent on the concentration used and the pathogen analyzed.

This study suggests that the action of the oil varies with concentration, opening opportunities for future research, such as in vivo analysis and the development of nanotechnological applications (nanocapsules with biodegradable polymers)

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