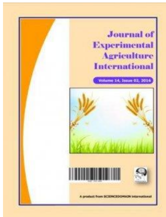

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# *In vitro* Fungitoxic Potential of Copaiba and Eucalyptus Essential Oils on Phytopathogens

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***Authors' contributions***

*This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** This study evaluates the *in vitro* fungitoxic effect of copaiba (*Copaifera* sp.) and eucalyptus (*Eucalyptus* sp.) essential oils on the mycelial growth of *Alternaria alternata* and *Colletotrichum musae*.
**Study Design:** The experiments comprised completely randomized designs with seven treatments and five replicates.
**Place and Duration of Study:** The work was carried out at the Center for Agrifood Science and Technology of the Federal University of Campina Grande, Pombal, Brazil, from July to August 2018.

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**Methodology:** Essential oils were incorporated into PDA culture medium (Potato-Dextrose-Agar) and poured into Petri dishes. The treatments consisted of four oil concentrations (0.4, 0.6, 0.8, and 1.0%), a negative control (0.0%) and two positive controls (the fungicides Thiram and Mancozeb). The fungi were inoculated on plates and incubated for seven days at 27±2°C. The diameter of the colonies was measured every day and use to calculate the percentage of mycelial growth inhibition (PGI) and index of mycelial growth speed (IMGS).
**Results:** Copaiba and eucalyptus oils reduced the mycelial growth of *A. alternata* and *C. musae* in all concentrations. The copaiba oil showed a moderate inhibition, with mean percentages ranging from 26.6 to 33.68% for *A. alternata* and 39.5 to 49.6% for *C. musae*. The eucalyptus oil showed high inhibition, with means ranging from 30.0 to 79.7% for *A. alternata* and 35.6 to 66.3% for *C. musae*. The concentrations 0.8 and 1.0% had the highest inhibition values in both oils treatments, but these inhibitions were lower than the ones caused by the fungicides. The eucalyptus oil at 1.31% could totally inhibit *A. alternata*, but in all other cases, the oils were unable to cause total inhibition.
**Conclusion:** Copaiba and eucalyptus oils inhibit the mycelial growth of *A. alternata* and *C. musae* under *in vitro* conditions. Concentrations of 0.8 and 1.0% provided the highest inhibitory effect.

*Keywords: Antifungal; Copaifera sp.; Eucalyptus sp.; fungitoxicity; mycelial growth.*

## 1. INTRODUCTION

Fruit production has great economic importance in Brazil, especially for the northeastern, southern, and southeastern regions, given that climatic conditions benefit the development of fruits [1]. Fruticulture contributes to the national economy both in the domestic market and exports with an annual production of 44 tons of fruit, a gross income of R$ 33 billion, and the maintenance of 5 million jobs [2].

Brazil is among the largest producers and exporters of food worldwide, being the third country in the ranking of fresh fruits production [3]. However, phytosanitary problems weaken the Brazilian international market of fruits, primarily due to the post-harvest diseases that reduce the final quality of fruits intended to export, which decreases production and entails significant losses [4].

Post-harvest infectious processes are latent. The phytopathogens establish in the pre-harvest stage when the fruits are still healthy. However, the symptoms of diseases appear only during the post-harvest, when the ripening favors the development of the pathogens. This process occurs mainly during the preparation of fruits for commercialization [5].

The fungi *Alternaria alternata* and *Colletotrichum musae* are among the main phytopathogens of post-harvest diseases. *A. alternata* causes post-harvest rot in papaya (*Carica papaya* L.) [6], melon (*Cucumis melo* L.) [4], blackberry *(Ribes nigrum* L.) [7] and other plants, and superficial spots on mandarin (*Citrus reticulata*) [8] and mango (*Mangifera indica* L.) [9]. *C. musae* is a widely distributed phytopathogen that causes anthracnose and crown rot disease [10,11,12,13], major post-harvest banana diseases (*Musa* spp.) in all producing regions of the world.

Brazilian farmers usually control the diseases caused by fungi using highly toxic chemicals that contaminate the environment and impair human health [1,14,15]. The use of pesticides limits the export of fruit due to the hard rules for the registration in demanding markets, such as European countries, and the residues left in food [16,17].

The search for natural products has increased worldwide, mainly due to problems caused by several synthetic products to the environment and human health [18]. New technologies and research have benefited complementary or alternative controls to plant diseases. In this sense, essential oils have several desirable properties when compared to other agrochemicals, such as larvicidal and fungicidal action [19,20], low risks to the environment and human health, and low cost [21].

The essential oils of eucalyptus (*Eucalyptus* spp.) and copaiba (*Copaifera* spp.) have shown antimicrobial activity in several studies. For example, eucalyptus essential oils showed fungitoxic capacity against *Fusarium oxysporum*, *Botrytis cinerea*, and *Bipolaris sorokiniana* [22], while the copaiba oil reduces the growth of *Colletotrichum gloeosporioides* [23], *Aspergillus spp*. [24] and *Scytalidium lignicola* [25].

The oil concentration interferes in its fungicidal and fungistatic activities. The same oil having a broad spectrum of action against different species of microorganisms may vary its minimum inhibitory concentrations for each species [26]. For example, eucalyptus oil (*Eucalyptus globulus*) has different minimum inhibitory concentrations according to the combated fungus [27].

In this sense, this study aimed to evaluate *in vitro* the fungitoxic effect of the essential oils of eucalyptus and copaiba on the mycelial growth of *Alternaria alternata* and *Colletotrichum musae*.

## 2. MATERIALS AND METHODS

### 2.1 Conduction of the Experiments

The study was carried out in the Laboratory of Phytopathology, Federal University of Campina Grande, Campus of Pombal-PB, between July and August of 2018. We developed four experiments, each one consisting of one type of essential oil against one species of fungus. The trials comprised completely randomized designs with seven treatments and five replicates each.

We used the 0878 strain of *Alternaria alternata* and 3499 of *Colletotrichum musae* provided by the collection of phytopathogenic fungi of Prof. Maria Menezes of the Federal Rural University of Pernambuco. The pure essential oils of copaiba and eucalyptus were purchased at a local store specialized in natural products.

The oils were added to autoclaved PDA culture medium (Potato Dextrose Agar), on the following concentrations (treatments): 0.0; 0.4; 0.6; 0.8, and 1.0%. The 0.0% treatment comprised a negative control. The positive controls consisted of supplementing the culture medium with two commercial fungicides, thiram and mancozeb, at the doses recommended by the manufacturers, 250 µL $L^{-1}$ and 0.2 g $L^{-1}$, respectively. The concentrations of the oils were chosen based on a study by Ugulino et al. [28].

After cooling, the medium was poured into Petri plates of 9 cm in diameter under aseptic conditions. Discs of culture medium with 1 cm in diameter containing the fungus mycelium were transferred to the center of each plate containing the treatments. Then, the plates were wrapped in plastic film and incubated for 7 days in a B.O.D incubator (Biochemical Oxygen Demand) at a temperature of 27±2°C.

The mycelial growth was assessed through daily measurements of colony diameters with a graduated ruler. For each colony, a daily data comprised the average of two perpendicular measurements. With the result of the measures, we calculated the percentage of mycelial growth inhibition (PGI; [29]) and the index of mycelial growth speed (IMGS; [30]), according to the formulas (1) and (2):

$$PGI = \frac{[(negative\ control\ growth - treatment\ growth)]\times 100}{negative\ control\ growth} \quad (1)$$

$$IMGS = \sum \frac{current\ mycelial\ growth - previous\ mycelial\ growth}{number\ of\ days\ of\ incubation} \quad (2)$$

### 2.2 Statistical Analysis of Data

The effect of oils concentrations on fungi growth was investigated through linear and quadratic regressions. Since in some cases the $R^2$ value was very close between the two models, we used the Akaike Information Criterion (AIC) to decide which model best represented the relationship between the oil concentrations and fungus growth. Simply put, the lower the AIC value, the better the model. Only the best models were plotted in graphs. The significance of each term of the equations was assessed using the test T.

Hypothesis tests were performed to verify the significance of differences between the treatment with the highest concentration of oils (1%) and the treatments with fungicides (thiram and mancozeb). We used Kruskal-Wallis non-parametric tests followed by Wilcoxon's multiple comparisons because the data did not satisfy the normal probability distribution assumption of parametric tests. All analyzes were carried out in the R 3.5.1 software [31]. Differences with a probability value below 5% were considered significant.

## 3. RESULTS AND DISCUSSION

All concentrations of copaiba and eucalyptus oils reduced the mycelial growth of *Alternaria alternata* and *Colletotrichum musae* (Fig. 1). The highest concentrations (0.8 and 1.0%) provided the best growth inhibitions. At these concentrations, respectively, copaiba oil inhibited 31.4 and 33.7% of *A. alternata* and 46.9 and 49.6% of *C. musae*, while eucalyptus oil inhibited 52.6 and 79.6% of *A. alternata* and 66.3 and 41.2% of *C. musae*.

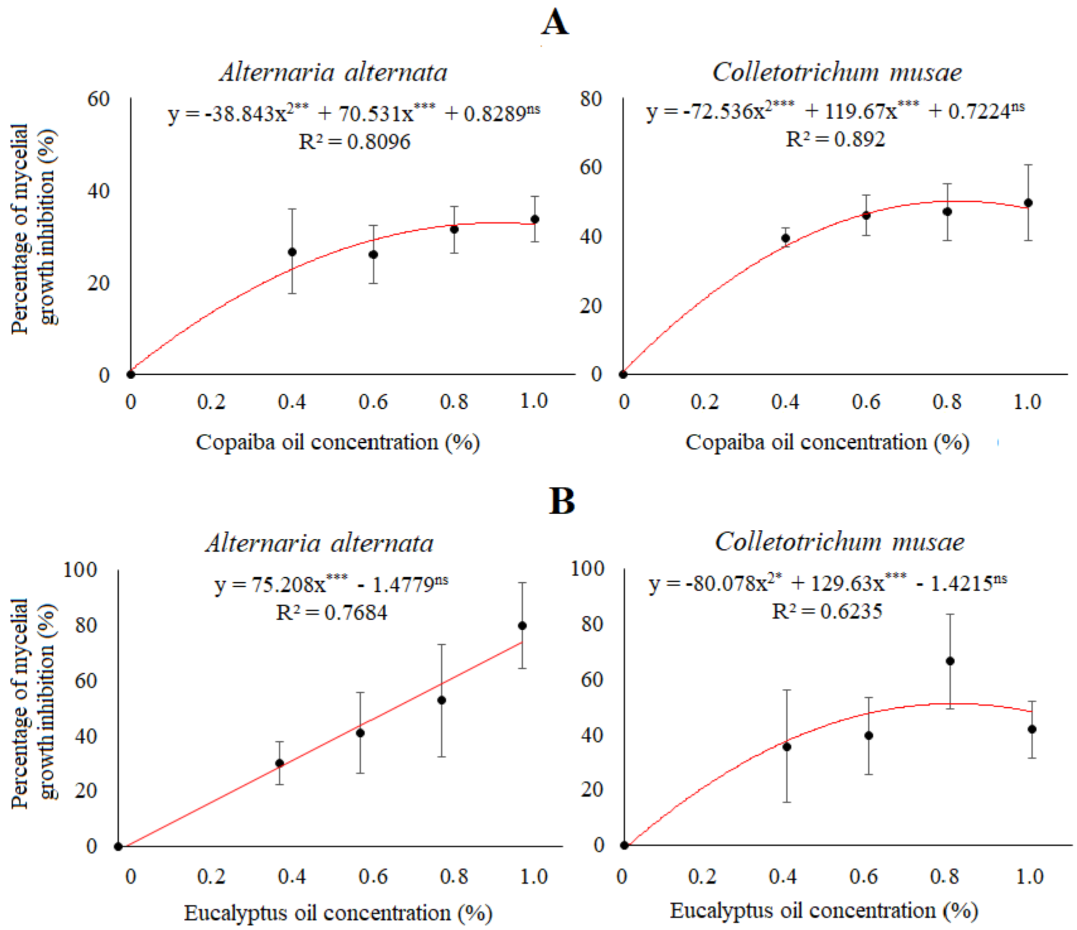


**Fig. 1. A. Effect of different concentrations of copaiba essential oil on the percentage of inhibition of mycelial growth of *Alternaria alternata* and *Colletotrichum musae*. B. Effect of different concentrations of eucalyptus essential oil on the percentage of inhibition of mycelial growth of *Alternaria alternata* and *Colletotrichum musae*. The red line shows the direction of the effect estimated by the regression analysis**

** P < 0.05; ** P < 0.01; *** P < 0.001; ns: No significant.*

*Copaiba oil: Alternaria alternata (F = 46.78, p = 1.191e-08); Colletotrichum musae (F = 90.82, p = 2.339e-11).*

*Eucalyptus oil: Alternaria alternata (F = 76.29, p = 9.205e-09); Colletotrichum musae (F = 18.22, p = 2.155e-05).*

Comparing the same concentrations, the inhibitions caused by both oils against *A. alternata* and *C. musae* differed from the inhibitions caused on other fungi. For example, copaiba and eucalyptus oils have a low fungitoxic effect on the growth of *Macrophomina phaseolina*. Ugulino et al. [28] reported the maximum inhibitions of 33 and 21%, respectively. On the other hand, these oils exert moderate to high inhibition on the growth of *Colletotrichum gloeosporioides*. Sousa et al. [23], also using the copaiba and eucalyptus oils, found reductions of 50 and 100% on the growth of *C. gloeosporioides*.

Copaiba and eucalyptus oils exhibited a dose-dependent effect when tested in the control of *A. alternata* and *C. musae*, i.e., inhibition increased with the dosage used. Ugulino et al. [28], when testing the effect of copaiba oil on the control of *M. phaseolina*, obtained the opposite result to ours, the highest inhibition occurred in the lowest dose tested (0.4%), with higher mycelial growth at higher oil concentrations. Thus, the increase of inhibitory power as a function of oil concentration depends on the species of microorganism studied. In some organisms, increased concentration potentiates the inhibitory effect, in others, the effect can be reduced, which generates waste of the product.

According to the regression results, the total inhibition could be reached only when using eucalyptus oil against *A. alternata* in a concentration of 1.31%. In the other cases, the modeling suggests that the oils are unable to inhibit 100% of mycelial growth. Therefore, we suggest that the maximum inhibition values were obtained among the concentrations tested in the present study. The use concentrations higher than 1.0% on the control of *A. alternata* and *C. musae* would be economically unviable.

In contrast to our results, tests made with oil from different species of eucalyptus [22] or different parts of the same plant [32] showed increasing inhibition with the rising of concentrations. Therefore, several factors can affect the chemical composition of the oil, which in turn influences its biological activity. Some examples are the genetic factors of the plant [33], ecological factors surrounding the plant and edaphoclimatic conditions (climate, temperature and soil type) [34]. The crop techniques applied to the vegetal material used to obtain the oil also affect its chemical composition, such as the period of harvest, the preparation of the material before the extraction, and the part of the plant used in the production of oil [35].

In the present study, the copaiba oil caused only a moderate inhibition on the mycelial growth of both fungi because it did not reach 50% inhibition. The eucalyptus oil caused a high inhibition because it reduced 79.6% of the growth of *A. alternata* and 66.3% of *C. musae*. França et al. [36], when evaluating the effect of peppermint oil on the growth of *A. alternata*, also obtained moderate inhibition, since the highest concentrations, 0.8 and 1.0%, inhibited only 41.6 and 37.1%, respectively. Other vegetable oils showed superior results, for example, the essential oil of mandarin (*Citrus reticulata*) inhibited 84% of *A. alternata* growth in the concentration of 0.1 mL 100 $mL^{-1}$, while at 0.2 mL 100 $mL^{-1}$ the oil caused total inhibition [37].

The essential oil of *Lippia gracilis* promoted complete inhibition of *Alternaria* sp. growth in the concentration of 750 µL $L^{-1}$ [6]. The essential oil of lemongrass (*Cymbopogon citratus*) had a minimum inhibitory concentration of 14.49 µg $mL^{-1}$ on the growth of *A. alternata* [38]. During *in vitro* tests to control *Colletotrichum musae*, the spiked pepper oil (*Piper aduncum*) inhibited 100% of mycelial growth and spore germination at concentrations of 100 µg $mL^{-1}$ and 150 µg $mL^{-1}$, while in the *in vivo* control of banana rot, the best inhibition was obtained with the oil in the concentration of 1.0% [13].

The antifungal effect of tested oils was generally lower than the obtained by commercial fungicides (thiram and mancozeb) (Fig. 2). In the control of *A. alternata*, the inhibition caused by copaiba oil had a weaker effect compared to the fungicides, whereas the essential oil of eucalyptus in the highest concentration had the same effect of the mancozeb fungicide and a lower effect than thiram fungicide. In the control of *C. musae*, the fungal inhibition promoted by both oils in the highest concentration was inferior to the two fungicides.

The mycelial growth speed of *A. alternata* and *C. musae* differed significantly between the concentrations of oils (Fig. 3). Treatments with concentrations of 0.8 and 1.0% reduced the growth rate of *A. alternata* for 0.44 and 0.43 cm $dia^{-1}$ using copaiba oil and 0.3 and 0.1 cm $day^{-1}$ using eucalyptus oil, both differing from the negative control, which had the highest growth rate (0.64 cm $day^{-1}$). Treatments at same concentrations above reduced the growth rate of *C. musae* to 0.62 and 0.59 cm $day^{-1}$ using copaiba oil and 0.41 and 0, 69 cm $dia^{-1}$ using eucalyptus oil, both differing from the negative control (1.18 cm $day^{-1}$).

The results obtained in the present study suggest the existence of biologically active compounds in the essential oils of copaiba and eucalyptus which guaranteed an antifungal activity against *Alternaria alternata* and *Colletotrichum musae* under *in vitro* conditions. This antimicrobial activity of the essential oils can be attributed to the presence of phenolic compounds and terpenoids in their composition [39].

Because of the high chemical complexity and variety of compounds present in essential oils, it is sometimes difficult to correlate their biological activities with isolated compounds. Their biological properties could be attributed to the synergism between these components or only to the major constituents [40,41,42].

The analysis of the chemical composition of different species of copaiba has evidenced β-caryophyllene as a major constituent [43,44,45], which was reported by Goren et al. [46] and Dahham et al. [47] for having significant antimicrobial activity. While the composition and concentration of constituents vary greatly among eucalyptus species [39,48,49,50]. Their biological activity depends on the type and nature of their constituents and also their respective concentrations in the oil [40]. In their study, Elaisse et al. [50] evaluated the antifungal activity of essential oil of 8 species of eucalyptus. The authors reported that the antifungal effect obtained was not related only to the major components (1,8-cineole), but to the synergistic effect obtained between the constituents at higher and lower concentrations.

The mechanisms of antifungal action of essential oils are poorly characterized, but in general they can be attributed to the chemical nature of their constituents. Freiesleben and Jäger [51] state that terpenes and phenolic compounds act directly on cell membrane rupture and inhibition of cell wall synthesis. Piper et al., [52] reported that essential oils increase the permeability of cell membranes, causing cellular leakage of essential macromolecules.

The low toxicity and the fast degradation in the environment give advantages to essential oils over the conventional agrochemical in phytopathogen control [53]. Also, the low cost of production and the reduction of risks to the health of producers and final consumers enhance the benefits of these products.

In this perspective, the results of this work provide information for the elaboration of natural products to agroecological crops aiming to reduce in the use of conventional fungicides. We suggest the study of *in vivo* control of *A. alternata* and *C. musae* to evaluate the activity of the oils on different plant species and to establish the safe inhibitory concentrations of these products.

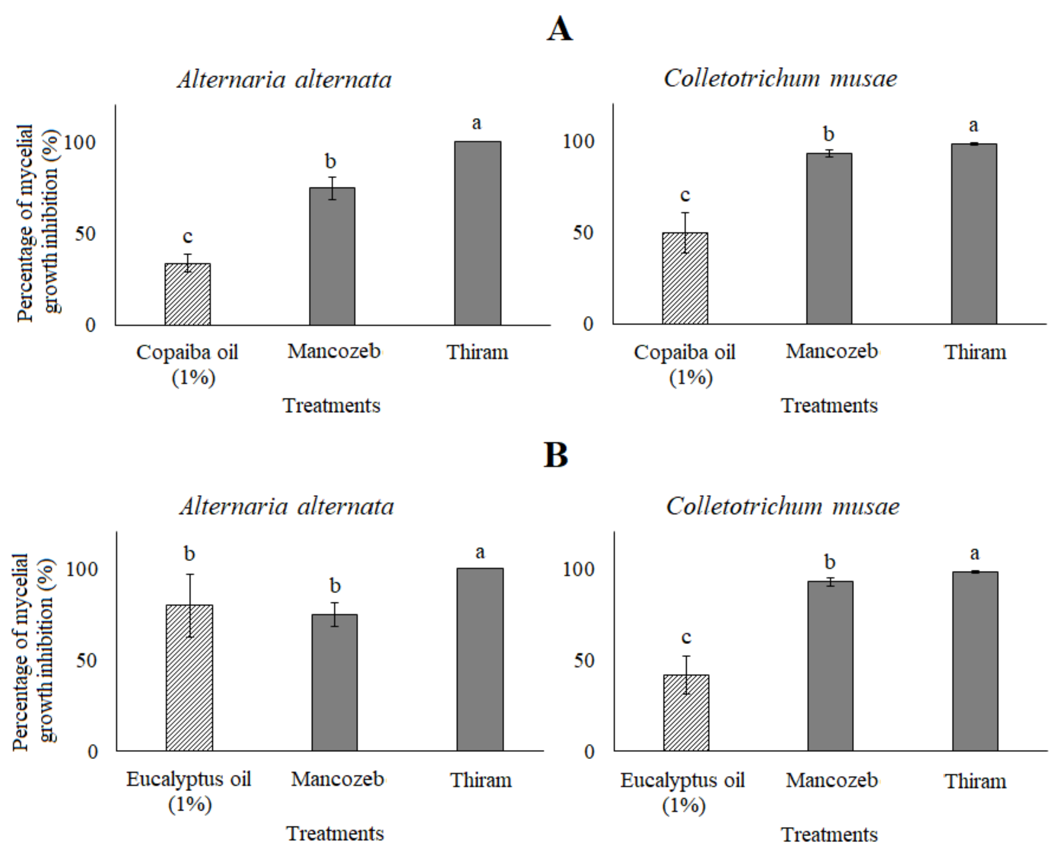


**Fig. 2. A. Percentage inhibition of mycelial growth of *Alternaria alternata* and *Colletotrichum musae* in the highest tested concentration of copaiba essential oil and the positive control treatments. B. Percentage inhibition of mycelial growth of *Alternaria alternata* and *Colletotrichum musae* in the highest tested concentration of eucalyptus oil and the positive control treatments**

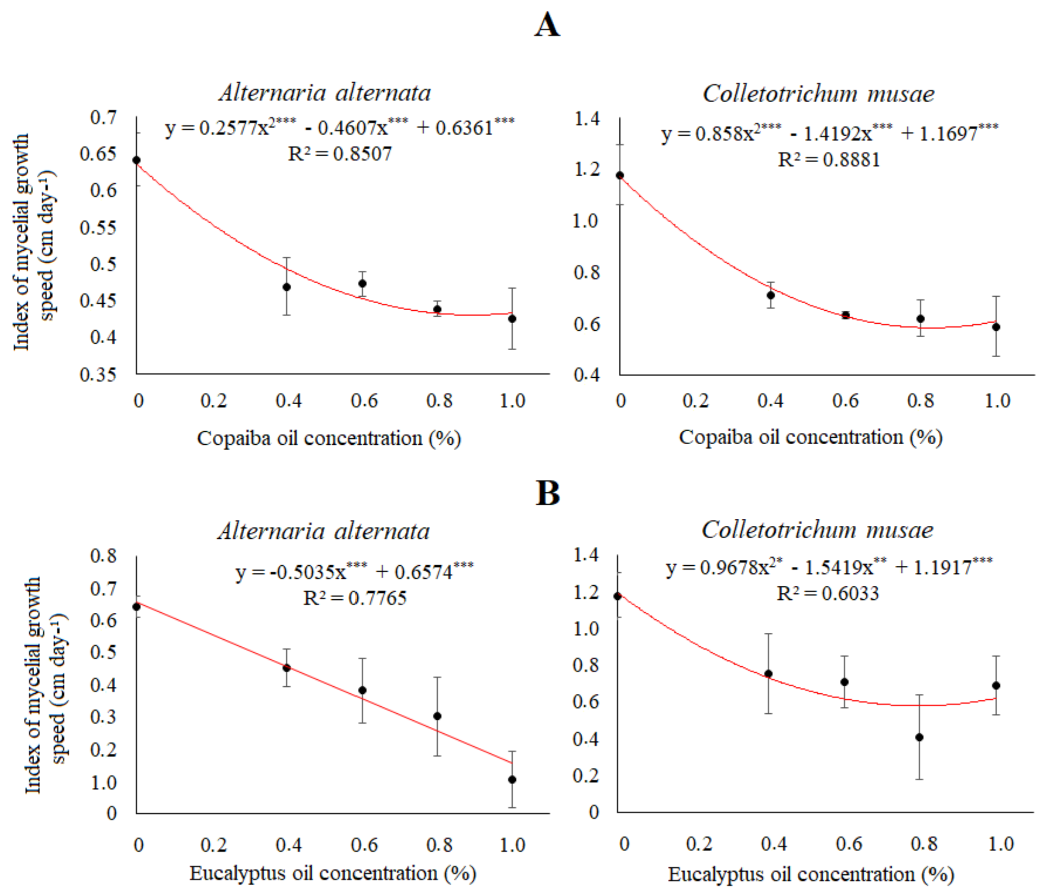


**Fig. 3. A. Effect of different concentrations of copaiba essential oil on the index of mycelial growth speed of *Alternaria alternata* and *Colletotrichum musae*. B. Effect of different concentrations of eucalyptus essential oil on the index of mycelial growth speed of *Alternaria alternata* and *Colletotrichum musae*. The red line shows the direction of the effect estimated by the regression analysis.**

**P < 0.05; ** P < 0.01; *** P < 0.001.*

*Copaiba oil: Alternaria alternata (F = 62.66, p = 8.242e-10); Colletotrichum musae (F = 87.32, p = 3.439e-11).*

*Eucalyptus oil: Alternaria alternata (F = 79.89, p = 6.081e-09); Colletotrichum musae (F = 16.73, p = 3.828e-05).*

## 4. CONCLUSION

The essential oils of copaiba (*Copaifera* sp.) and eucalyptus (*Eucalyptus* sp.) inhibit the growth of *Alternaria alternata* and *Colletotrichum musae* under *in vitro* conditions. Concentrations of 0.8 and 1.0% promoted the highest inhibitory effect.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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