Agricultura Familiar:
Pesquisa, Formação e Desenvolvimento
RAF. v.18, nº 01 / jan-jun 2024, ISSN 1414-0810 / E-ISSN 2675-7710

ÓLEOS ESSENCIAIS DE PLANTAS NO BIOCONTROLE IN VITRO DE *COLLETOTRICHUM MUSAE* E *COLLETOTRICUM GLOEOSPORIOIDES*

PLANT ESSENTIAL OILS IN THE BIOCONTROL IN VITRO OF COLLETOTRICHUM MUSAE AND COLLETOTRICHUM GLOEOSPORIOIDES

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Resumo

Em virtude das severas perdas na produção brasileira de frutos e do mau uso de agrotóxicos tem-se buscado novas técnicas de controle alternativo de doenças fúngicas em plantas, como é o caso do emprego de óleos essenciais. Diante disso, o objetivo deste trabalho foi avaliar o potencial antifúngico in vitro dos óleos essenciais de hortelã (*Mentha piperita* L.), alecrim (*Rosmarinus officinalis* L.) e melaleuca (*Melaleuca alternifolia* Cheel.) frente os fungos fitopatogênicos *Colletotrichum musae* e *Colletotrichum gloeosporioides*. O trabalho foi realizado na Universidade Federal do Maranhão, onde foram realizados testes com os óleos essenciais, avaliando-se a inibição total ou parcial do crescimento micelial deste fitopatógeno, dependendo do óleo essencial utilizado. Os resultados indicaram que todos os óleos testados apresentaram determinado grau de efeito inibitório, porém óleo essencial de melaleuca demonstrou maior efeito inibitório do crescimento micelial dos fungos *C. musae* e *C. gloeosporioides* quando comparado aos óleos de hortelã e alecrim.

Palavras-chave
Fruticultura, Sustentabilidade; Produtividade; Controle biológico.

Abstract

Owing to the severe losses in Brazilian fruit production and the wrongful use of agrochemicals, there has been a search for new techniques of alternative controls of fungal diseases in plants, such as the use of essential oils. This paper aimed to evaluate the *in vitro* antifungal potential of essential oils extracted from peppermint (*Mentha piperita* L.), rosemary (*Rosmarinus officinalis* L.) and tea tree (*Melaleuca alternifolia* Cheel.) against the phytopathogenic fungi *Colletotrichum musae* and *Colletotrichum gloeosporioides*. The study was conducted at the Federal University of Maranhão, where tests were carried out with essential oils to evaluate the total or partial inhibition of mycelial growth in the phytopathogens depending on the essential oil used. Results indicated that all essential oils analyzed had a certain degree of inhibitory effect, although the tea tree oil caused the strongest inhibition on the mycelial growth of *C. musae* and *C. gloeosporioides* when compared to the peppermint and rosemary oils.

Keywords
Fruticulture, Sustainability; Productivity; Biological control.

INTRODUCTION

One main issue related to fruit production, on a global scale, is disease manifestations in the post-harvest stage. Such diseases include anthracnose, which is caused by fungi of the *Colletotrichum* genus (Silva *et al*., 2009). These fungi can affect both mature and immature fruits by directly penetrating the cuticle. Among the vast variety of fungi, *Colletotrichum* is considered one of the most important genera related to plant pathogenicity, especially the species (Penz.) Penz. & Sacc. and C. musae (Berk. & M.A. Curtis) Arx. (Michereff *et al*., 2005; Bonett *et al*., 2012).

C. gloeosporioides is the causative agent with the highest incidence in several fruit species. It occurs in nearly all fruit-producing regions in Brazil and in many other countries, affecting plants' leaves, flower buds, tendrils, branches, and fruits (Nery-Silva *et al*., 2007a). Causing similar damage, *C. musae*, the causative agent of anthracnose in bananas, considerably hinders the *in natura* commercialization and consumption of this fruit (Silva, Cordeiro, 2000; Ploetz *et al*., 2003).

Against this background, strategies for the control of diseases such as anthracnose are necessary to guarantee healthier plants and, consequently, a higher production and productivity of the plant species being harvested (Nozaki *et al*., 2013). Currently the control of this pathogen is carried out through chemical use. However, the indiscriminate use of these products has been causing several problems, such as food residues, environmental contamination (soils and groundwater), intoxication of rural workers, the mortality of natural enemies and pollinators, and also the reduction of microorganisms populations, affecting biological balances, making fruits more susceptible to reinfestation and infestation by other pathogens (Paiva *et al*., 2021; Carvalho *et al.,* 2008). In addition, the use of these products can generate resistance, harming the environment (Lozada *et al*., 2019). Therefore, these products are now under criticism by different stakeholders, which has motivated the search for new molecules that can be used to control diseases (Paiva *et al*., 2021; Carvalho *et al*., 2008).

Concern for the development of alternative control methods has grown, especially among organic producers, whose market has grown significantly. In production systems where the use of chemical control is not allowed, there is a need for alternative methods, with proven efficacy (Viana *et al.,* 2020).

Essential oils have received increasing attention as potential alternatives to commercial pesticides in crop protection, given their promising biological properties against plant pathogens, pests and weeds (Camele *et al*., 2019, Raveau *et al*., 2020, Viana *et al*., 2020). Considering all these aspects, studies have been carried to find specific non-toxic essential oils with antifungal effects (Almeida, 2015).

Among medicinal plants, the tea tree (*Melaleuca alternifolia* Cheel), small tree or bush of Australian origin with needle-like leaves and yellow or purplish flowers, has been used for years in the treatment of diseases (Siani *et al.*, 2000; Hoare, 2010). Another prominent species is the peppermint (*Mentha piperita* L.), an aromatic species belonging to the Lamiaceae family, which has a menthol-rich essential oil, a substance responsible for its characteristic refreshing flavour (Sousa *et al*., 2012, David *et al*., 2007). Rosemary (*Rosmarinus officinalis* L.) is also among the many plant species with medicinal potential. Rosemary is a perennial herb native to the Mediterranean, and is cultivated worldwide (Yu *et al.,* 2013). According to Rašković *et al*. (2014), Rosemary essential oil has antioxidant and antimicrobial properties, and is used in the food preservation industry, in aromatherapy and to make fragrances.

From this perspective, our study aimed to evaluatie the *in vitro* antifungal potential of peppermint, rosemary and tea tree essential oils against the phytopathogenic fungi *Colletotrichum musae* and *C. gloeosporioides*, which are the causative agents of anthracnose in a variety of fruit plants.

METODOLOGY

The study was conducted at the Plant Physiology Lab at the Federal University of Maranhão, Dom Delgado Campus, in São Luís, MA.

The essential oils used were extracted from peppermint (*Mentha piperita* L.), tea tree (*Melaleuca alternifolia* Cheel.) and rosemary (*Rosmarinus officinalis* L.) These were produced and acquired commercially from the Amantikir Origem Natural® company, located in the city of São Lourenço, MG, Brazil.

As for fungi, isolates from *C. gloeosporioides* and *C. musae* were used in the experiments, obtained from damaged tissues (direct isolation) of infected papaya and banana fruits, respectively. Isolates were preserved and deposited in the fungi collection of the Research Group on Microbial Diversity and Ecology of the Federal University of Maranhão.

Essential oils were directly diluted in a melting PDA (Potato Dextrose Agar) culture medium, in a vertical laminar flow bench (model Pa-50 Eco, Pachane Ltda), as to obtain the following concentrations: 0.1; 0.2; 0.3; 0.5 and 1% for peppermint and tea tree, and 0.25; 0.5; 0.75 e 1% for rosemary. Media with extracts were poured in Petri dishes of 90 mm diameter. All operations were performed in sanitized conditions.

The medium containing different oil concentrations was poured in Petri dishes, and the inoculum, consisting of a 5-mm diameter mycelium disk of fungi cultivated for 10 days in PDA medium, was put at the centre of each Petri dish, which was kept at 25±2 ºC, with a 12-hour photoperiod. As treatment control, fungi were cultivated in dishes containing only the culture medium.

Mycelial growth evaluation (mm) was verified by the daily measuring of colonies' diameters obtained from the average of two diametrically opposed measures, over the course of 10 days or until the colony reached the dish's edge. Mycelial growth inhibition (%) was calculated through the equation: GI (%) = [(Dc - Dt) / Dc] × 100, where Dc (mm) is the colony diameter of the control treatment and DT (mm) is the colony diameter of the essential oil treatment. The mycelial growth rate index was calculated through the equation: MGRI = ∑(D - Db) / N, where D= current average colony diameter, Db= average colony diameter on the day before, N= number of days since inoculation.

A completely randomized experimental design was used, with three replicates per treatment. Data relative to daily growth were subject to an analysis of variance and regression tests. Data on GI and MGRI were compared through the Tukey test at the 5% probability level.

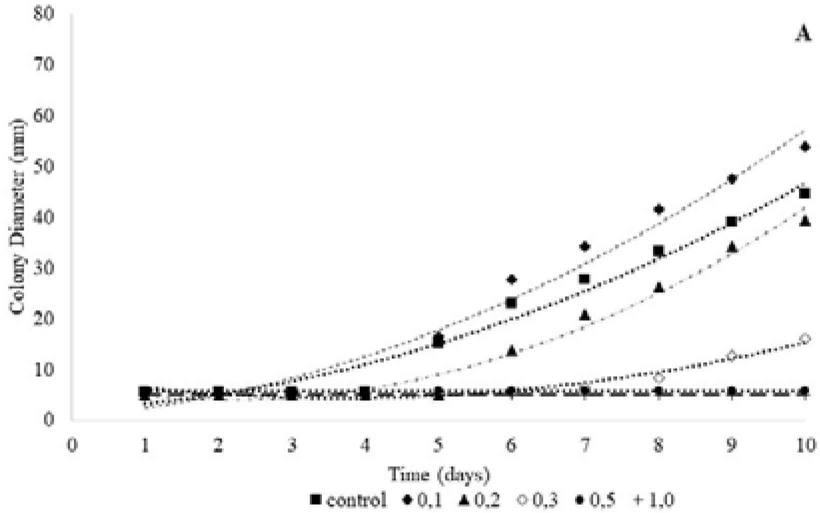
RESULTS AND DISCUSSION

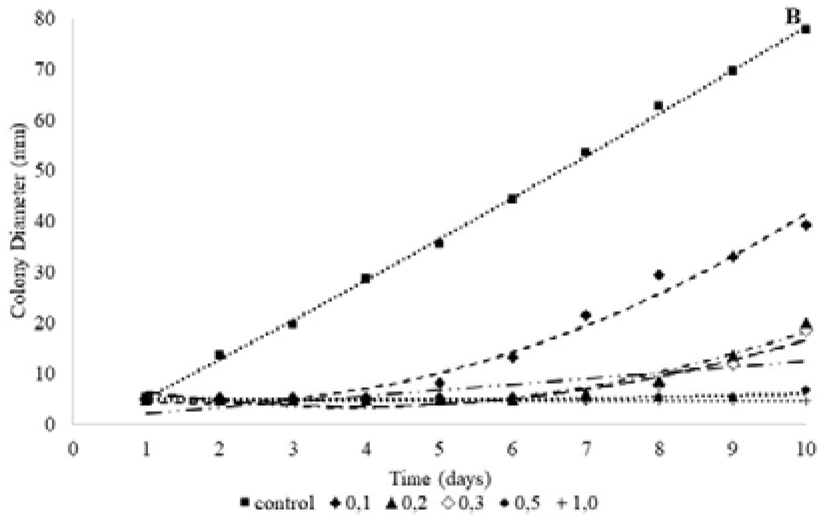
Treatments with peppermint oil (*M. piperita*) caused a mycelial growth reduction of *C. gloeosporioides* in concentrations above 0.1% (Figure 1a). With 0.2%, 0.3% and 0.5% of oil added to the culture medium, a fungistatic effect was observed, with a mycelial growth inhibition (GI) of 11.41%, 64.22% and 87.16%, respectively, compared to the control treatment (Table 1).

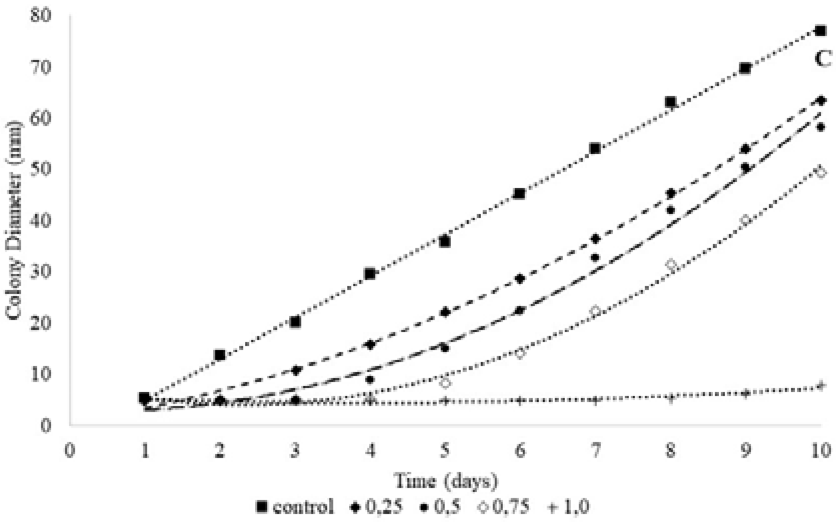
The tea tree essential oil (*M. alternifolia*) reduced mycelial growth of *C. gloeosporioides* when compared to the medium with no tea tree oil. The oil's inhibitory effect increased along with its concentration (Figure 1b). The highest mycelial growth inhibition was obtained at a concentration of 1%, with a 94.24% inhibition, thus showing that this dosage yields an antifungal property against *C. gloeosporioides*. At concentrations of 0.1%, 0.2%, 0.3% and 0.5%, it was possible to observe a fungistatic effect, with GIs of 49.39%, 74.36%, 76.12% and 91,52%, respectively, when compared to the control treatment (Table 1).

The concentration of 1% presented a fungicide effect, with a GI of almost 95% (Table 1), hindering the mycelial growth of the fungus. As for the rosemary essential oil (*R. officinalis*) (Figure 1c), the concentrations of 0.25%, 0.50%, 0.75% and 1% inhibited the mycelial growth of *C. gloeosporioides* in 17.58%, 24.39%, 36.09% and 89.93%, respectively, when compared to the control treatment.

Figure 1: Effect of oils from *Mentha piperita* L. (A), *Melaleuca alternifolia* Cheel. (B) and *Rosmarinus officinalis* L. (C) in the in vitro mycelial growth *of C. gloeosporioides*.







In *C. musae*, the tea tree essential oil exerted an inhibitory effect on mycelial growth, with a strongest reduction at the concentration of 1% (Figure 2b). All concentrations of this essential oil yielded a fungistatic effect, with an inhibition of 9.6%, 23.90%, 37.88% and 90.63% at the concentrations of 0.1%, 0.2%, 0.3% and 0.5%, respectively (Table 1).

For the peppermint essential oil, there was an 87.73% inhibition at the 0.5% oil concentration (Table 1), thus showing a high fungistatic potential until the 9th day, while the 1% oil concentration showed a fungicide effect with a 95.07% mycelial growth inhibition during the 10 days of the test (Table 1 and Figure 2 A). The 0.2% and 0.3% concentrations presented a fungistatic effect, not causing changes in fungi growth during the first 4 days. The fungus started to develop only after this period, remaining smaller than the control treatment until the end of the assessment (Figure 2a). Tests with Rosemary oil were not efficient in reducing *C. musae* mycelial growth at concentrations of 0.25, 0.5 and 0.75% (Figure 2c). Inhibition occurred only at the 1% concentration (GI = 19.75%) when compared to the control (Table 1).

For all of the oils used, the mycelial growth of *C. gloeosporioides* was inversely proportional to the increase in oil concentrations (Figure 3A, 3B and 3C). Because of the almost equal values for concentrations 0.5 and 1% observed in peppermint and tea tree oils, the regression curve showed a quadratic fitting. As for the rosemary oil, values were adjusted into a line (linear regression). The same behaviour occurred for *C. musae*, except for the rosemary oil, where mycelial growth in treatments 0.25%, 0.50% and 0.75%

exceeded that of the control treatment (Figure 3D, 3E and 3F).

Figure 2: Effect of oils from *Mentha piperita* L. (A), *Melaleuca alternifolia* Cheel. (B) and *Rosmarinus officinalis* L. (C) in the in vitro mycelial growth of *C. musae*.

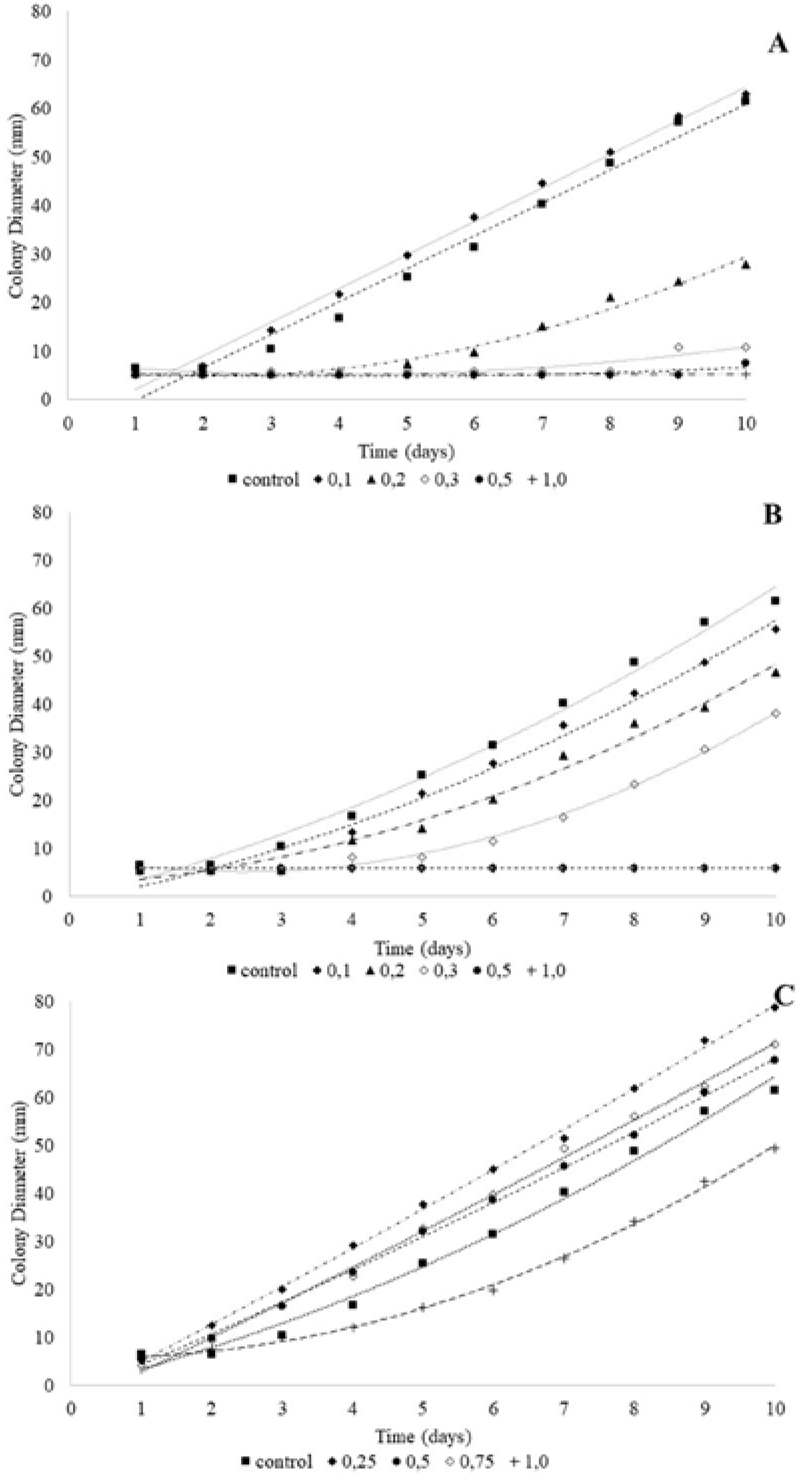
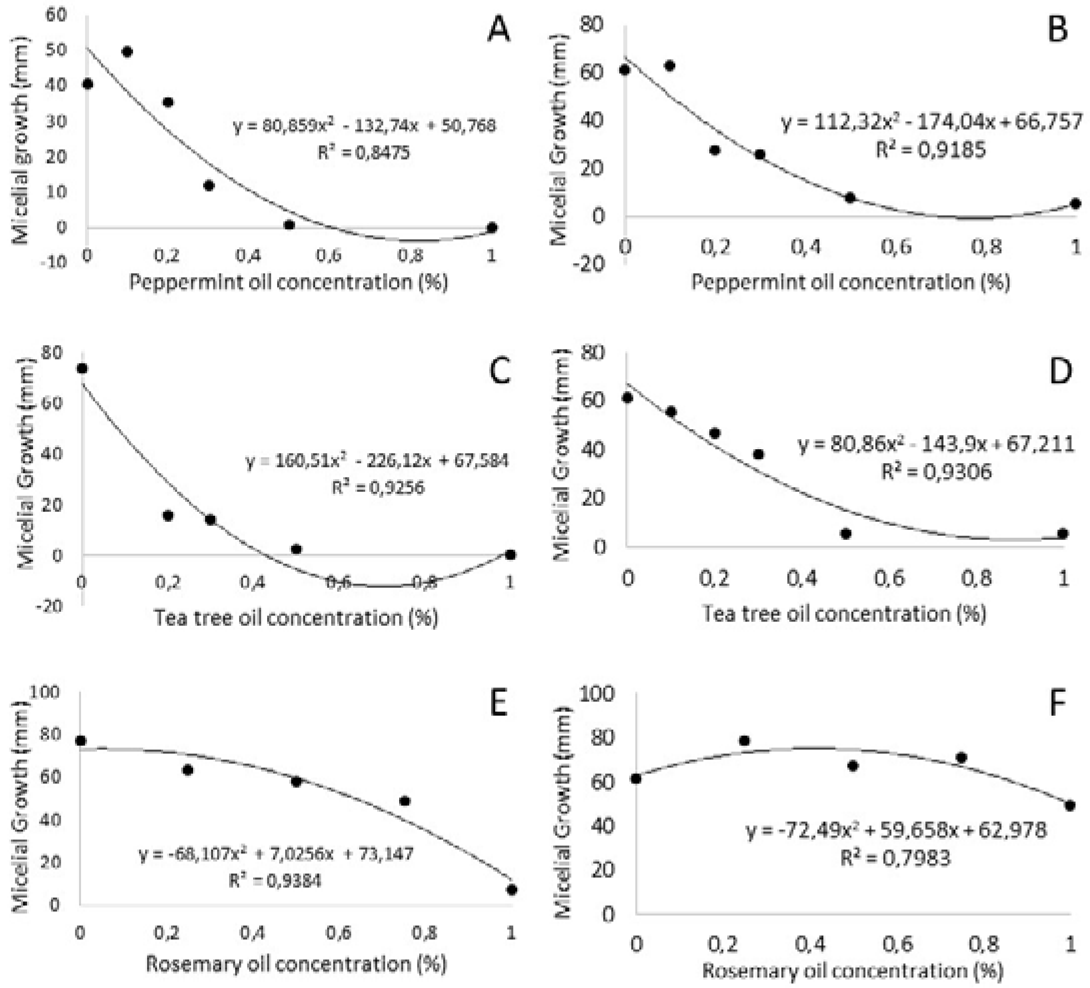


Table 1: Mycelial growth inhibition index (%) of *C. gloeosporioides* and *C. musae* by essential oils of peppermint (*Mentha piperita* L.), tea tree (*Melaleuca alternifolia* Cheel.) and rosemary (*Rosmarinus officinalis* L.)

		Fungi	
	Oil concentration	*C. gloeosporioides*	*C. musae*
Peppermint	0.1	-20.55b	-2.47a
	0.2	11.41b	54.67a
	0.3	64.22a	57.43b
	0.5	87.16a	87.73a
	1.0	88.81a	91.57a
Tea tree	0.1	49.39a	9.60b
	0.2	74.36a	23.90b
	0.3	76.12a	37.88b
	0.5	91.52a	90.63a
	1.0	94.24a	90.63a
Rosemary	0.25	17.58a	-28.17b
	0.50	24.39a	-10.01b
	0.75	36.09a	-15.50b
	1.0	89.93a	19.76b

Averages followed by equal letters in the line do not differ from each other based on the Tukey test ($\alpha \leq 0.05$).

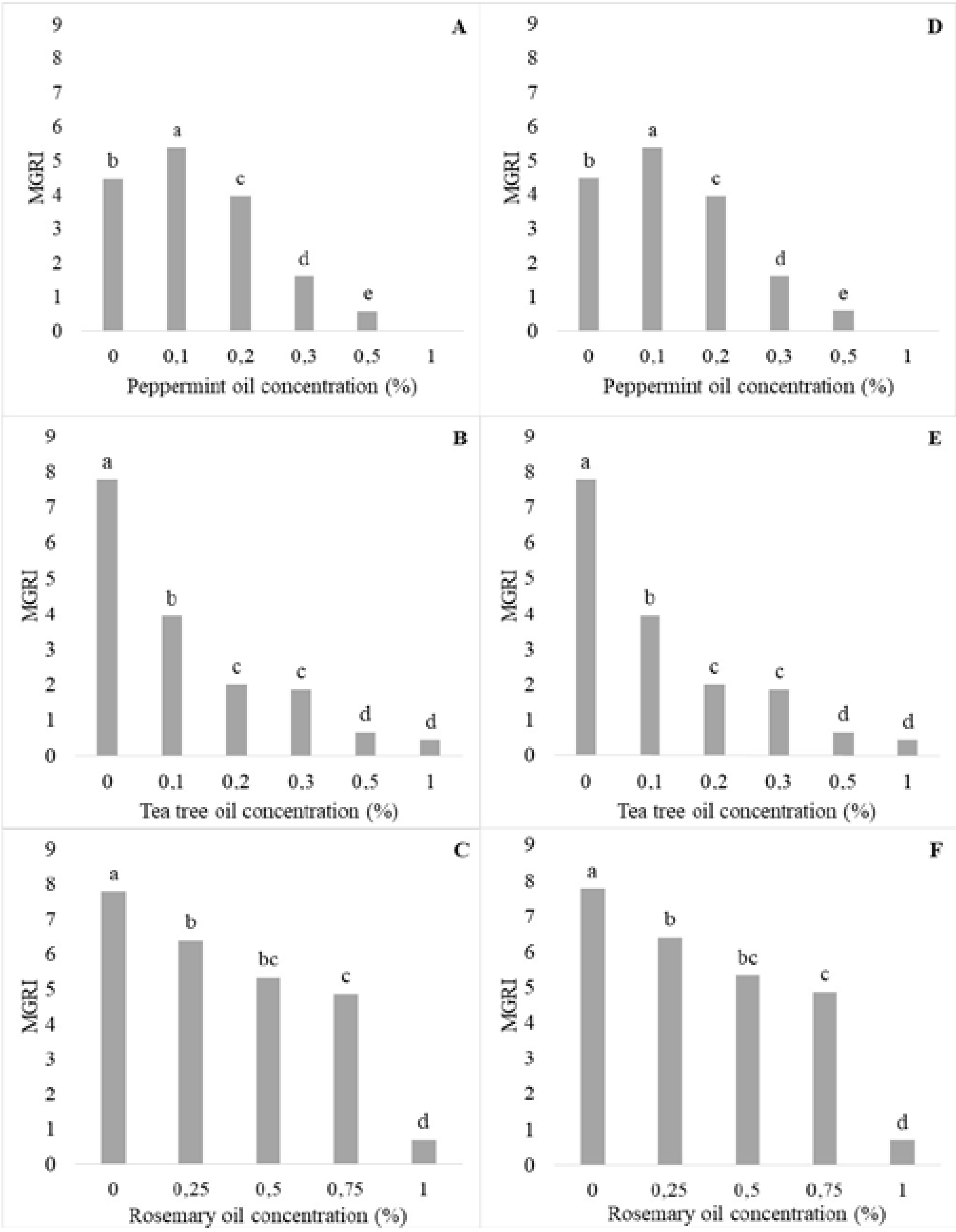
Figure 3: Mycelial growth of *C. gloeosporioides* (A, B and C) and *C. musae* (D, E and F) in different concentrations of Peppermint (A and D), Tea tree (B and E) and Rosemary (C e F) essential oils.



Regarding the mycelial growth rate index (MGRI) of *C. gloeosporioides*, a significant relationship was found between growth rate and oil concentrations, in which the tea tree had a higher MGRI in control dishes, while treatments of 0.1%, 0.2%, 0.3%, 0.5% and 1% concentrations showed a gradual reduction in growth rates as oil concentrations increased (Figure 4B). A similar result was observed for the rosemary oil, where at the concentration of 1% the lowest rate of mycelial growth was observed (Figure 4C). With the peppermint oil, an inversely proportional relationship between MGRI and oil concentrations was also noticed: from 0.2% onwards, as oil concentration increased, growth rates decreased (Figure 4A).

For *C. musae*, a reduction in MGRI as tea tree oil concentration increased was observed, figuring as a gradual and significant decrease in each of the tested concentrations, with the lowest MGRI of 0.44 obtained for the 1% concentration (Figure 4E). For the peppermint oil, the MGRI of the 0.1% concentration did not differ from the control, exhibiting a considerable decrease starting from the 0.2% concentration (Figure 4D). In contrast, for the Rosemary oil, only the maximum concentration of 1% caused a significant reduction in the growth rate of this fungus species (Figure 4F).

Figure 4: Mycelial Growth Rate Index (MGRI) of *C. gloeosporioides* (A, B and C) and C. musae (D, E and F) in different concentrations of Peppermint (A and D), Tea tree (B and E) and Rosemary (C and F) oil.



There is a growing need to develop methods for the control of diseases in cultivated plants (especially fruits consumed *in natura*), which should be efficient and less aggressive for both the environment and human health. Based on that need, the in vitro antifungal potential of peppermint, rosemary and tea tree essential oils was tested against fungi that cause anthracnose in papaya and banana fruits (*C. gloeosporioides* and *C. musae*, respectively).

Among these potential biocontrol agents, the tea tree essential oil was the most efficient one against the pathogens tested. In this study, the 1% oil concentration was the best dose among the ones tested, resulting in the complete inhibition of mycelial growth in the studied fungi species. Similar results were obtained by Scheuermann *et al*. (2011), when testing the fungicide activity of this same oil in the same concentration on the biocontrol of *Bipolaris oryzae*, a rice phytopathogen.

Tea tree oil concentrations below 1% exerted an inhibitory activity on the growth of *C. gloeosporioides* and *C. musae*, thus being considered fungistatic starting from the concentration of 0.1%, the lowest concentration tested. Martins *et al*. (2010) tested the tea tree essential oil on the control of *Macrophomina phaseolina, Sclerotinia sclerotiorum* and *Alternaria alternata*, and observed an inhibitory effect starting from the concentration of 0.2%. Other studies, such as Hammer *et al*. (2004) and Barbosa *et al.* (2015), also observed the intense fungistatic and fungicide activity of the tea tree essential oil against the fungus species *Candida albicans*. Melaleuca essential oil also significantly reduced mycelial growth of the fungi *Lasiodiplodia theobromae, Macrophomina phaseolina, Sclerotinia sclerotiorum* and *Alternaria alternata* in concentrations above 0,2%, suggesting that the oil may be useful in the treatment of fungal infections in plants (Martins *et al*., 2010, Viana *et al*., 2020).

The main components of the tea tree oil are the terpinen-4-ol, with antimicrobial activity, and the 1,8-cineol, which is irritating to the skin (Castelo *et al*., 2013). Regarding the action mechanisms of the tea tree oil, studies suggest that it acts directly on fungi's respiratory enzymes, leading to an inhibition of the respiratory process, which makes it an extremely functional oil (Hofling *et al*., 2019).

Exhibiting a fungistatic effect at low concentrations, the tea tree oil represents an economic and ecologically viable alternative to the control of plant diseases in the post-harvest phase, slowing down the growth of fungus species in fruits. By doing that, it increases the shelf life of fruits such as banana, papaya and mango, which are the main targets of anthracnose caused by *Colletotrichum* in Brazil. The use of the tea tree oil would aid in filling the gap related to the controls and requirements regarding the use of biological products that do not pose any risks to humans or the environment (Tatagiba *et al.,* 2002; Santos *et al*., 2018).

Regarding the peppermint essential oil, the best mycelial growth inhibition rates were observed in *C. gloeosporioides* when compared to *C. musae*. Such an inhibitory effect can be observed in both species starting from the 0.2% oil concentration. Studies such as Souza *et al.* (2012) present tests at different concentrations, showing the peppermint oil's fungicide and fungistatic potential against different *Colletotrichum* species. These results are like those of the present study, where a marked reduction in mycelial growth rates was observed as the peppermint oil concentration increased.

Tyagi & Malik (2011) found antimicrobial activity in the peppermint oil against fungi isolates of *Penicillium digitatum, Aspergillus flavus, Fusarium oxysporum,* and *Colletotrichum gloeosporioides.* Other studies, such as the ones by Silva *et al*. (2012) and Barbieri (2019), show that the peppermint essential oil, at the concentration of 100%, was able to inhibit the development of different fungi species, such as *Aspergillus* sp., *Penicillium rubrum, Sclerotinia* sp., and *Fusarium verticillioides*. These results demonstrate the oil's potential of effectively fighting organisms that act as pathogens to different plant species.

According to Santos (2011), this antimicrobial property is granted by the presence of menthol and mentone, the most abundant components of peppermint leaves. However, this property can vary according to the plant's sample origin and the fungus species.

Tests carried out in this study with the Rosemary essential oil yielded different results depending on the fungus species being tested. In *C. gloeosporioides*, a dose-dependent effect was observed, with inhibition even at low concentrations and a reduction in MGRI as Rosemary oil concentration increased. As for *C. musae*, inhibition was only observed at the concentration of 1%. This can be explained by the fact that the fungitoxic potential can be associated not only with the oil composition, but also with the pathogen's sensibility to one or more compounds in different quantities. It should be noted that the concentration of active principles is not uniform during a plant's life cycle, varying according to the habitat, harvest, and preparation (Roswalka, 2014).

According to Genena *et al.* (2008), the most abundant component found in Rosemary is the isocarnosol, which, together with other compounds, such as the α-pinene, bornyl acetate, camphor and carvacrol, is responsible for the plant's antimicrobial activity. Santos (2016), when testing the Rosemary oil on the in vitro control of the fungus *Sclerotium rolfsii*, obtained a reduction in mycelial growth starting from the 0.2% concentration. Other studies noted that this oil was able to inhibit the mycelial growth of *Alternaria carthami, Alternaria* sp. and *Rhizoctonia solani* (Hillen *et al*., 2012) and reduce the mycelial growth of *Phomopsis sojae*.

For nearly all the oils tested here, we were able to observe a dose-dependent relationship for cases in which as oil concentrations increased, a gradual inhibition of the phytopathogen occurred, as well as a decrease in the MGRI (Mycelial Growth Rate Index).

The use of essential oils on the alternative control of anthracnose constitutes a broad field to be explored, especially for species of the *Colletotrichum* genus, which just as many other phytopahogens are at high risk of acquiring resistance to conventional fungicides (Ramos, Andreani, 2016). Furthermore, the mechanism of action of essential oils which exhibit some kind of biological activity is not very clear. Many studies suggest that cell membranes of microorganisms are the targets of bioactive volatile compounds, that can be cause degradation of the cell wall, disruption of cytoplasmic membrane e cell lysis (Dias, Miranda, 2020).

Currently, this study yielded important information regarding the antifungal effect of some essential oils, opening new perspectives to the continuity of studies in the field of biodiversity and bioprospection. The search for promising plant species as sources for the development of new essential oils with antifungal potential is especially relevant considering the increase in microorganisms' resistance to the synthetic fungicides currently available (Chaves *et al.,* 2018). Little is known about the effects of these essential oils on the great diversity of phytopathogenic fungi that affect economically important crops. Such information can contribute to the alternative control of some diseases and the future development of new products.

FINAL CONSIDERATIONS

Research results show that the essential oils extracted from Peppermint (*Menta piperita* L.), Rosemary (*Rosmarinus officinalis* L.) and the Tea tree (*Melaleuca alternifolia* Cheel.) exerted a fungicide and fungistatic effect at certain concentrations. Considering the need less environmentally aggressive agricultural practices especially for small fruit producers, the use of essential oils is a promising alternative, requiring investments in research that prove their effectiveness in the field and in the postharvest phase. Therefore, their use is a viable and sustainable alternative for the control of anthracnose.

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