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Inhibition Effect of Vegetable Oils on the Mycelial Growth of *Macrophomina phaseolina* (Tassi.). Goid

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Abstract

Macrophomina phaseolina (Tassi.). Goid is the etiological agent of one of the major diseases in cowpea bean crop, commonly known as gray rot stem. Due to the lack of registered phytosanitary products to control this disease, the search for alternative control methods is increasingly common. The objective of this study was to evaluate the effect of essential oils on mycelial growth of *M. phaseolina*. The oils tested were *Mentha* sp., *Eucalyptus* spp., *Copaifera* sp., and *Lippia gracilis* at concentrations of 0.4; 0.6; 0.8 and 1.0%. The work was conducted in Phytopathology Laboratory of the Federal University of Campina Grande. Daily measurements of the colony diameter were performed in two perpendicular directions until it filled the entire surface of the culture medium of one of the plates. The experiment was conducted in a completely randomized experimental design in a factorial arrangement 4 × 4 + 1 + 1, with sixteen treatments plus one negative control and one positive control, which consisted of the supplemental application in the medium (BDA) of the fungicide Sportak 450 EC (Procloraz), five replications, totaling 90 experimental plots. The data were interpreted through non-parametric analysis of variance and the means were compared by the Kruskal-wallis test, with a 5% probability of error and Scott Knott at 5% probability in cases where there were significant differences and data normality. The *Mentha* sp. essential oil and *Lippia gracilis* showed better results in inhibiting mycelial growth, while the *Eucalyptus* essential oil and *Copaifera*, although potentially promising, showed intermediate inhibition of fungal mycelial growth.

Keywords: *Copaifera* sp., *Eucalyptus* sp., *Lippia gracilis*, *Mentha* sp.

1. Introduction

Macrophomina phaseolina (Tassi.). Goid is a fungus that lives in the soil and spreads throughout the world using seeds as hosts. This pathogen causes severe crop losses through a series of diseases such as seedling blight, pre-emergence damping-off, stem rot, basal stem rot, root rot, and charcoal rot (Gupta, Sharma, & Ramteke, 2012; Menezes, Machado, Silveira, & Silva, 2013). Due to its high saprophytic ability and the development of microsclerotia, which are resistance structures able to remain in the soil for years, *M. phaseolina* also shows a high survival capacity, even when subjected to adverse conditions.

This fungus affects a wide range of hosts, being pathogenic for more than 680 botanical species (Farr, Rossman, Palm, & Mccray, 2008). Several cultivated species with commercial value are among the *M. phaseolina* hosts, for example: cotton (*Gossypium hirsutum*, L.), sweet potato (*Ipomoea batatas*, L.), cowpea beans (*Vigna*

unguiculata, L.), common bean (*Phaseolus vulgaris*, L.), melon (*Cucumis melo*, L.), watermelon (*Citrullus lanatus*), maize (*Zea mays*), and soya (*Glycine max*, L.) (Michereff, Andrade, & Menezes, 2005).

Conventional control for this pathogen comprises the use of agrochemicals, and, due to the continuous and indiscriminate use of fungicides, the emergence of resistant populations became a common problem, besides the high toxicity to human health and environment. Thus, the development of less harmful techniques to control the phytopathogens arises as a valuable tool to protect both the human health and the environment. Among these techniques, the natural products used to control pathogens has gained attention in the agribusiness (Fonseca et al., 2015).

An alternative to control of pests and diseases is the use of essential oils with low harmful effects (Ootani et al., 2013). These compounds are complex mixtures and may contain up to 60 chemical components distributed at various concentrations, but with two or three molecule kinds dominating the compound. They may play an essential role in protecting plants against disease-causing bacteria, fungi, and insects (Bakkali, Averbeck, Averbeck, & Idaomar, 2008). The success of these oils relates to their ability to dissolve in lipid media, allowing a harmony between the oil and the lipid components present in the cells of the pathogens, modifying the cell structure (Brum, 2012).

Many vegetal extracts and oils succeeded in control the mycelial growth of several fungi. Silva, Teixeira, Santos, and Pessoa (2012), evaluating the antifungal activity of vegetal extracts of clove, garlic, pepper, and neem, observed the inhibition of *Colletotrichum gloeosporioides*, *Fusarium oxysporum* f. sp *vasinfectum*, and *Pyricularia oryzae*. The essential oils of *Eucalyptus citriodora* and *Azadirachta indica* reduce the mycelial growth of *Colletotrichum acutatum* in strawberry (Dias-Arieira et al., 2010), and the peppermint (*Mentha piperita* L.) essential oil inhibits the mycelial growth of numerous fungi (Freire, 2006).

Taking in mind the benefits of non-toxic compounds as surrogates of toxic agrochemicals, in this study, we analyze the antifungal effect of the essential oils on the *in vitro* growth of *Macrophomina phaseolina* (Tassi.). Goid. For the tests, we used oils produced from *Eucalyptus* sp., *Mentha* sp., *Copaifera* sp., and *Lippia gracilis* (hereafter referred only by the genus names).

2. Matherials and Methods

2.1 Laboratory Structure and Fungus Isolation

The experiment was carried out at the Phytopathology Laboratory of the Center for Agri-Food Science and Technology (CCTA) of the Federal University of Campina Grande (UFCG), Pombal, Brazil, from December 2016 to March 2017. The fungus *Macrophomina phaseolina* (Tassi.). Goid was obtained from cowpea roots with symptoms of gray stem rot.

2.2 Obtaining the Essential Oils

The oils of *Eucalyptus* and *Mentha* were purchased in natural products stores. The *Copaifera* oil was bought at a street fair in the state of Pará, Brazil. The oil of *Lippia* was obtained through the steam distillation technique, which was carried out on the II Laboratory of Biology of the Biology Department at the State University of Rio Grande do Norte.

2.3 Treatments and Experimental Design

We applied a completely randomized experimental design, with sixteen treatments plus one negative control and one positive control (factorial design: 4 × 4 + 1 + 1), with five replications each. The treatments consisted of the Potato Dextrose Agar (PDA) culture medium supplemented with the four essential oils (*Eucalyptus*, *Mentha*, *Copaifera*, and *Lippia*) in four concentrations (0.4; 0.6; 0.8; and 1%). Also, we set the negative control (without essential oil supplementation) and the positive control (supplemented with 750 μL L^{-1} of the fungicide Prochloraz, which is the dosage indicated by the producer). The concentrations of essential oils were set according to previous studies (Sousa, Serra, & Melo, 2012).

2.4 Experimental Procedures

The oils were added to PDA culture medium after autoclavation at 120 °C. Under an aseptic condition, the media were poured on Petri dishes (90 × 15 mm). After the medium solidification, we placed, in the center of each plate, an inoculum comprised of a discoidal sample of culture medium with 8mm in diameter containing 7-days old *Macrophomina phaseolina*. The dishes were incubated at 27±2 °C until at least one colony cover all area of a plate.

The mycelial growth was recorded daily through the measuring of the colony diameter in two perpendicular directions. With the average of the two diameters, we calculated the percentage of mycelial growth inhibition

(*PGI*) and the index of mycelial growth speed (*IMGS*) according to the Equations 1 and 2 (Edginton, Khew, & Barron, 1971; Oliveira, 1991; respectively):

$$PGI = \frac{Negative\ control\ growth - Treatment\ growth}{Negative\ control\ growth} \times 100 \quad (1)$$

$$IMGS = \sum \frac{Current\ mycelial\ growth - Previous\ mycelial\ growth}{Number\ of\ days\ of\ incubation} \quad (2)$$

2.5. Statistical Analysis

To assess the effect of treatments on the *PGI* and *IMSG*, we used the Kruskal-Wallis test, a non-parametric analysis of variance. When significant differences were found (*Eucalyptus*, *Mentha*, and *Copaifera* oils), we applied the Scott-Knott test to verify which treatments differed from the others. We used linear and non-linear regression models to analyze the behavior of the effect of increasing oils concentration on the mycelial growth of *M. phaseolina*. For all tests, we recognized significant differences at a level of 5% error probability.

3. Results and Discussion

For all treatments, the percentage of mycelial growth inhibition (*PGI*) and the growth rate index (*IMGS*) of *M. phaseolina* showed high compatibility on the last day of incubation. The *Lippia gracilis* oil showed the best results regarding inhibition of fungus growth and mycelial growth speed, with significant differences compared to the negative and positive controls. At all concentrations, the *Lippia* oil completely prevented mycelial growth (*PGI* = 100%; *IMGS* = 0.00), showing better results even when compared to the fungicide (*PGI* = 78.53%; *IMGS* = 0.27; Table 1).

Table 1. Percentage of mycelial growth inhibition (*PGI*) and index of mycelial growth speed of *Macrophomina phaseolina* under different concentrations of essential oils (*Eucalyptus*, *Mentha*, *Copaifera*, and *Lippia*) and fungicide. Pombal, Paraíba, Brazil, 2017. The values are the average of five replicates

Essential oils and controls	Concentrations (%)	[1]*PGI* (%)	[2]*IMGS*
Negative control	0.0	0.00f	1.97a
Mentha sp.	0.4	33.95d	1.29c
	0.6	42.11d	1.11c
	0.8	52.27c	0.88d
	1.0	54.48c	0.82d
Eucalyptus sp.	0.4	5.10f	1.93a
	0.6	9.77f	1.83a
	0.8	8.83f	1.85a
	1.0	21.01e	1.58b
Copaifera sp.	0.4	33.72d	0.38e
	0.6	25.93e	0.43e
	0.8	22.50e	0.46e
	1.0	20.40e	0.47e
Lippia gracilis	0.4	100.00a	0.00g
	0.6	100.00a	0.00g
	0.8	100.00a	0.00g
	1.0	100.00a	0.00g
Positive control (fungicide)	750 µL L^{-1}	78.53b	0.27f
CV (%)	-	17.02	19.45

Note. In the columns, mean values followed by the same letter did not differ by Scott-Knott test, assuming a significance level of $p < 0.05$.

[1] Kruskal-Wallis result: H = 86.305; H-crit = 27.587; $p < 0.001$.

[2] Kruskal-Wallis result: H = 84.294; H-crit = 27.587; $p < 0.001$.

The inhibition caused by the plants of *Lippia* genera relates to the high contents of carvacrol (41.7%) and thymol (10%), which have antimicrobial activity (F. J. A. Matos, M. E. O. Matos, Machado, & Craveiro, 2004). These chemical compounds reduce the conidial germination and cause the fungus death (Demetzos, Perdetzoglou, &

Tan, 2001; Oliveira, Terao, Carvalho, Innfecco, & Albuquerque, 2008). Although several monoterpenoids show antimicrobial properties, the level of toxicity of the different compounds varies according to the species of combated fungus. However, the carvacrol and thymol are effective against most fungi (Muller, Berger, & Yegen, 1995; Tsao & Zhou, 2000). These molecules provoke lesions on the cell membrane and the consequent cell death (Pina-Vaz et al., 2004; Isman & Machial, 2006).

The complete success of the *Lippia* oil during our *in vitro* tests suggests the need to test lower doses in future experiments to set a minimum concentration for cost-effective use. Furthermore, some studies show an antibacterial effect of the *Lippia* oil (Nascimento, Carvalho, Furtado-Neto, Martins, & Vieira, 2007), creating opportunities to test this oil against phytopathogenic bacteria. Thus, the oil seems to have a nonspecific effect on the control of pathogens, which is a highly desirable feature for the natural agrochemicals.

The oils of *Mentha* and *Copaifera* also differed significantly from the negative control at all concentration levels (Table 1), suggesting the occurrence of an antifungal effect. However, they were less efficient than the fungicide on both the growth inhibition (maximum *PGI* of 54.48 and 33.72%, respectively) and the reduction of mycelial growth speed (minimum *IMGS* of 0.82 and 0.38, respectively). The *Eucalyptus* oil had the weakest inhibition effect, differing from the negative control only at 1.0% concentration (*PGI* = 21.01; *IMGS* = 1.58). Perini et al. (2013) also found low inhibition of the mycelial growth of *P. grisea* by the *Eucalyptus* and *Copaifera* oils (30.0 and 20.39%, respectively).

Although the *Mentha* and *Eucalyptus* oils showed weaker antifungal effects than the *Lippia* oil and the fungicide, their inhibition increased with the dosage (Figure 1). Perhaps, the use of concentrations higher than 1% may increase the percentage of inhibition and make these oils more effective. For example, El-Mougy, El-Gamal, and Abdalla (2008) reported that *Mentha* essential oil at 4.0% has a total inhibitory effect on the mycelial growth of *M. phaseolina*. Also, Dawar, Younus, Tariq, and Zaki (2007), using the aqueous extract of leaves, stem, bark, and fruit of *Eucalyptus* sp., reported the increase of inhibition upon *M. phaseolina* with the rise in extract concentrations.

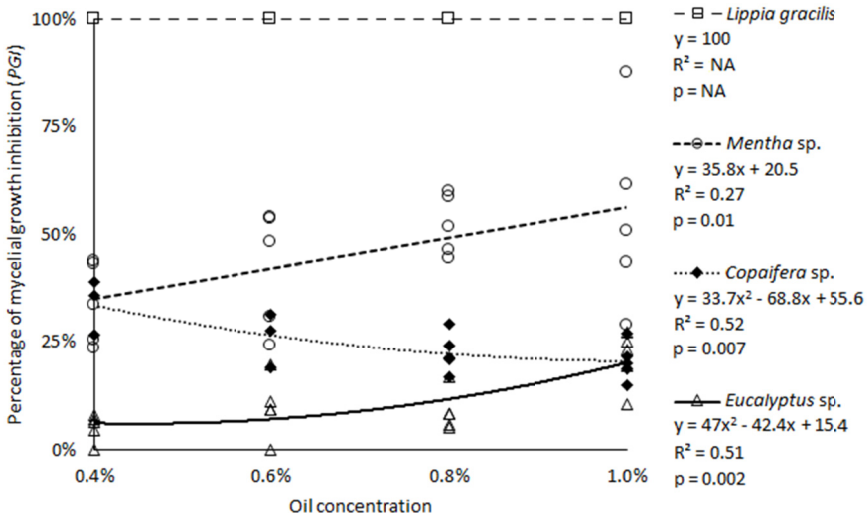

Figure 1. Percentage of mycelial growth inhibition of *Macrophomina phaseolina* as a function of different concentrations of the vegetal essential oils. The lack of variance among the *Lippia* oil results precluded the estimates of R^2 and p-value for this regression

The antimicrobial action of the *Mentha* oil is caused by the presence of menthone, piperitone oxide, α-terpineol, carvone, and linalool, among which, the menthol stands out with the primary antimicrobial activity (Hussain, Anwar, Nigam, Ashraf, & Gilani, 2010; Nascimento, Carvalho, Warnick, Palheta, & Santos, 2013). Singh, Muftah, and Belkheir (2015), Tyagi and Malik (2011), and Abdel-Kader, El-Mougy, and Lashin (2011) also reported the inhibitory action of *Mentha* on phytopathogenic fungi. Some compounds present in this plant have several advantages upon synthetic products, for example, the low toxicity and the fast degradation in the environment (Coimbra, Soares, Garrido, Sousa, & Ribeiro, 2006).

The main antifungal compounds in *Eucalyptus* oil are citronellal, krypton, cuminaldehyde, and phellandral, which are present mainly in the leaves (Guenther, 1950). Venturoso, Bachi, and Gavassoni (2011) showed a reduction of 23% in mycelial growth of *Didymella bryoniae* due to the addition of *Eucalyptus* oil. However, the above results differ from Fiori et al. (2000), in which the oil *Eucalyptus citriodora* at 20 µl caused a total inhibition of *D. bryoniae* mycelial growth. According to Salgado et al. (2003), several kinds of *Eucalyptus* essential oils differ in their antifungal potential. Gilles, Zhao, and Agboola (2010) attribute the antimicrobial activity of essential oils to the presence of phenolic and terpenoid compounds. Thus, the *Eucalyptus* oils activity depends on the type and concentration of these two components, which vary among the species, season, location, climate, leaves age, and method of extraction (Pereira, 2010). Also, the accelerated increasing behavior of the model adjusted to this oil (Figure 1) suggests the need to test concentrations above 1%, that may substantially raise the percentage of inhibition or stabilize the mycelial response.

On the other hand, the *Copaifera* oil decreased its effect with the increasing concentration and stabilized at the highest concentrations (quadratic model; Figure 1), which suggests that the 0.4% level is enough to promote the mycelial inhibition. The inhibitory effect of *Copaifera* seems to depend on the fungal species under control. For example, Sousa, Serra, and Melo (2012) and Ishida, Amaral, Gurgel, Tremacoldi, and Sousa-Filho (2008) verified that the inhibitory potential of *Copaifera* upon *Colletotrichum gloeosporioides* and *Fusarium solani* raised with the increase in oil proportion. Conversely, Deus, Alves, and Arruda (2011) found that changing concentrations of the oil did not affect the inhibition of *Aspergillus flavus*. Although the compounds of the *Copaifera* oil have been reported as antifungal against several phytopathogens (Dawar, Younus, Tariq, & Zaki, 2007), there is still scarce information in the literature regarding the most efficient way to use this oil and its most suitable concentrations.

Finally, numerous studies report the inhibitory potential of several essential oils on different fungal species (*e.g.*, Akgul & Kivanc, 1988; Ribeiro & Bedendo, 1999; Sousa, Serra, & Melo, 2012; Carnelossi, Scuwan-Estrada, Cruz, Itako, & Mesquini, 2009; Khaledi, Taheri, & Tarighi, 2015). At the tested concentrations, our results show the low efficiency of *Eucalyptus*, *Mentha*, and *Copaifera* oils in contrast to high efficacy of *Lippia* on the control of *M. phaseolina*. The *Lippia gracilis* oil stands out from the others, even that reported on literature, due to its overwhelming efficiency at minimal concentrations.

In this perspective, the results of this work can be applied to the elaboration of natural phytosanitary products that can be implemented in agroecological crops aiming at the substitution or reduction in the application of conventional fungicides. For this, future studies should be carried out to control *M. phaseolina* in vivo, in order to evaluate the activity of the oil on different plant species of commercial value and to establish safe inhibitory concentrations of the product.

4. Conclusions

(1) The *Lippia gracilis* essential oil showed the best inhibitory effect on the mycelial growth of *M. phaseolina*. It reached 100% of inhibition in all the concentrations, significantly exceeding the effectiveness of the fungicide.

(2) The *Mentha*, *Eucalyptus,* and *Copaifera* oils inhibited *M. phaseolina* at weaker levels than the *Lippia* and the fungicide. However, the *Mentha* and *Eucalyptus* raised their effects with the increase in concentrations, while the *Copaifera* oil decreased the inhibition with the rise in concentrations.

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