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Antifungal Activity of Melaleuca Essential Oil Against *Lasiodiplodia theobromae* in Maize Seeds

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A B S T R A C T

The fungus *Lasiodiplodia theobromae* causes black rot in maize seeds, leading to losses in crops production. The fungicides used in the control of black rot are highly toxic, requiring the development of less harmful control techniques. This work evaluates the effect of Melaleuca essential oil against *L. theobromae*, both under *in vitro* conditions and on maize seeds. The *in vitro* experiment compared the growth of the fungus in PDA culture medium containing the oil at 0.25, 0.50, 075, and 1.0%, a negative control, and positive control (Thiram fungicide). The *in vivo* experiment analyzed the incidence of *L. theobromae* in maize seeds treated with the essential oil at 0.50, 0.75, and 1.00%, and in the negative and positive controls. After the treatments of seeds, we inoculated them with the fungus and performed sanity tests. In the *in vitro* test, Melaleuca essential oil inhibited 100% of mycelial growth in the 0.75% concentration. In the *in vivo* test, the oil at 1.0% concentration reduced the incidence of fungus in the seeds to 45%, being more effective than the synthetic fungicide. Thus, Melaleuca oil has a strong potential as a control agent against fungus *L. theobromae* in maize seeds.

Introduction

Brazil is the third-largest producer of maize (*Zea mays* L.) in the world. The 2016/2017 crop covered a cultivated area of 17.6 million hectares (USDA, 2017). The sum of the productivity of the first and second national crop reached 97.8 million tons and obtained an average production of 5.6 tons per hectare (Conab, 2018). Although highly productive, maize crops are susceptible to several pathogens, and fungi are the microorganisms that promote the most severe losses in production (Barboza, 2015; Fancelli and DouradoNeto, 2008). Grain losses in Brazil comprise 10% of the harvest, resulting from factors such as climate and diseases, as well as machine regulation errors or limitations of

manual harvesting. In the post-harvest phase, losses occur due to improper storage, reducing the quantity and quality of stocked products (IBGE, 2005).

Countless fungal species attack maize seeds, damaging plant establishment, weakening seedlings, and decreasing population. The occurrence of pathologies impairs grain yield, quality, palatability, and nutritional value (Pinto, 1998; Wordell Filho and Casa, 2010). For example, the fungus *Lasiodiplodia theobromae* causes black rot in maize seeds. Symptoms begin with punctate discoloration in the ear top grains, which then turn brown and finally black and dry, presenting a dry rot (Embrapa, 1980; Oliveira *et al.,* 2013; Ma *et al.,* 2016).

Traditionally, producers control black rot using commercial fungicides based on Carboxanilide (Carboxin), Dimethylditio-carbamate (Tiram) and Ethylene Glycol. However, the long-term use of these pesticides harms human health and the environment due to toxicity and pollution caused by chemical wastes. Besides, new control agents become necessary over time due to the selection and multiplication of resistant microorganisms (MAPA, 2009; Silva *et al.,* 2013; Araújo *et al.,* 2017; Miranda *et al.*, 2017).

The problems with synthetic fungicides motivate the search for less harmful natural products to control phytopathogens. The essential oils, which are plant secondary metabolites, stands out among fungicides produced from plants (Venturoso *et al.,* 2011; Araújo and Costa, 2013).

Essential oils are an affordable option to replace or supplement traditional chemical treatments because they have low toxicity to humans (Nardelli *et al.,* 2009; Vigan, 2010) and high efficiency on *in vitro* and *in vivo* control of phytopathogens, including in seed treatment (Médice *et al.,* 2007; Pereira *et al.,* 2008; Rodrigues *et al.,* 2006; Silva and Bastos, 2007).

The essential oil of Melaleuca (*Melaleuca alternifolia*), also called tea tree oil, has in its composition terpinen-4-ol, which has antifungal, bactericidal, antiviral, anti-inflammatory, anesthetic, analgesic, antineoplastic, insecticidal, and antiparasitic properties. Several studies addressing traditional medicine and phytopathogen control report the antimicrobial activity of this oil (Lis-Balchin *et al.,* 2000; Hammer *et al.,* 2003; Caldefie-Chezet *et al.,* 2006; Baldissera *et al.,* 2014; Souza *et al.,* 2015).

Some researches show that the Melaleuca oil has low toxicity in humans and mammals (Mikus *et al.,* 2000; Souza and Fernandes, 2006; Nardelli *et al.,* 2009; Vigan, 2010). The Australian Chemical Component Standardization Committee established that the oil should contain amounts below 15% of cineol, which is irritating to the skin, and terpenen-4-ol above 30%, to ensure minimal antimicrobial efficacy (Simões *et al.,* 2002).

This study aims to evaluate the effect of Melaleuca oil at various concentrations on the development of *L. theobromae* both under *in vitro* conditions and on maize seed disinfection. Our results will support the development of oil-based technologies to control several fungal pathologies in maize crop, reducing toxicological risks, and improve production sustainability.

Materials and Methods

The study was carried out at the Phytopathology Laboratory of the Federal University of Campina Grande - UFCG, at Pombal Campus, Paraíba, from June to December 2018.

The *Lasiodiplodia theobromae* strain (CMM 4534) was supplied by the Prof. Maria Menezes Phytopathogenic Fungi Culture Collection of the Federal Rural University of Pernambuco. This phytopathogen was isolated from typical fruit lesions. The fungus was multiplied in the PDA (potato dextrose agar) culture medium and incubated at 27 ±2°C.

Melaleuca (*Melaleuca alternifolia*) essential oil was purchased from a health food store. The company informed the chemical constituents of the essential oil through technical reports. Terpinen-4-ol (42%) and Gammaterpinene (22%) comprised the primary components.

In vitro experiment

The *in vitro* experiment comprised a completely randomized design with six treatments in five replications each. Treatments included one positive control (PDA culture medium supplemented with 1mL L^{-1} of Thiram fungicide), one negative control (no supplementation), and four treatments containing increasing doses of Melaleuca essential oil (0.25, 0.50, 0.75 and 1.0%). The oil concentrations were adapted from Concha *et al.,* (1998) and Martins *et al.,* (2010) with the objective of testing doses similar to those found in these researches.

We added the oil and fungicide to the sterilized culture medium under aseptic conditions. The media was poured into 75x15mm Petri dishes. After solidification, one disc of culture medium (Ø = 1 cm) containing seven-day-old fungus was deposited in the center of each plate. Plates were incubated at 27±2°C to stimulate mycelial development. Two perpendicular diameters of each colony were measured daily until one of them reached the plate margins. With the results of the measurements, we calculated the percentage of mycelial growth inhibition (PGI; Bastos, 1997) and the mycelial growth rate index (IMGS; Oliveira, 1991) according to formulas (1) and (2):

$$PGI = \frac{[(negativecontrolgrowth - treatmentgrowth)]\times 100}{negativecontrolgrowth} \quad (1)$$

$$IMGS = \sum \frac{currentmycelialgrowth - previousmycelialgrowth}{numberofdaysofincubation} \quad (2)$$

In vivo experiment (maize seeds)

Five hundred seeds of AG 1051 hybrid maize were disinfected by immersion in 2% sodium hypochlorite solution for 5 minutes. Afterward, the seeds were washed three times with autoclaved water and dried at room temperature. The *in vivo* experiment consisted of five treatments with ten repetitions each. Treatments comprised solutions of 100 ml of autoclaved distilled water and the following treatments: Melaleuca oil at concentrations of 0.5%, 0.75%, and 1.00%; one positive control (100 mL L^{-1} of Thiram fungicide); and one negative control. Each solution received the addition of 100 µL of Tween 80 to aid dilution of the products in water. One hundred corn seeds were immersed in each solution for 5 minutes and then dried on germitest paper at room temperature for one hour.

The fungus *L. theobromae* was grown in plates containing PDA culture medium and incubated in a BOD incubator at 27 ±2°C for eight days. After treatments, seeds were contaminated with fungus by deposition on mycelia, so that all seeds remained in contact with fungal structures for 32 hours. Inoculation technique was adapted from Ramos *et al.,* (2014).

After being treated and inoculated, the seeds were subjected to the sanity test by filter paper method with freezing (Limonard, 1966). A hundred seeds of each treatment were distributed in 10 Petri dishes (Ø = 14 cm). Ten

seeds were placed equidistantly on each plate containing two sheets of filter paper previously moistened with sterile distilled water and incubated at 27 ±2°C. After 24 hours, the plates were transferred to a freezer at -20ºC for 24 hours and then to a BOD incubator for a further five days. Finally, the number of fungal colonies was counted in each plate, and the results expressed as percentage of infected seeds.

Statistical analysis

The effect of oil concentration on fungal growth was analyzed applying regressions in quadratic plateau model for *in vitro* experimental data and in the quadratic model for *in vivo* experiment. Differences between the effects of Melaleuca oil and synthetic fungicide on *Lasiodiplodia theobromae* growth and percentage of infection were compared by the Mann-Whitney test (nonparametric pairwise comparisons) assuming a significance level of 5%. The analyses were performed using the R Core Team 3.5.1 program.

Results and Discussion

In vitro experiment

The Melaleuca essential oil reduced the mycelial growth and growth rate of *Lasiodiplodia theobromae* at all tested concentrations. Inhibition percentages increased significantly with the oil dosage until reaching the maximum value (PGI = 100%) at 0.75 and 1.0% (Figure 1A). The mycelial growth rate decreased with increasing oil concentration until reaching the minimum value (IVCM = 0.0 cm day^{-1}) also at 0.75 and 1.0% (Figure 1B). The inhibition caused by the essential oil at its highest concentrations was similar to the synthetic fungicide. Thus, under *in vitro* conditions, the essential oil may substitute the commercial synthetic fungicide.

The compound Terpinen-4-ol comprises most of the Melaleuca oil, which also contains other chemical components, such as α-terpinene, γ-terpinene, and 1,8-cineole, which contribute to its potent antifungal activity. The mechanisms of action of this essential oil include lipid peroxidation, inhibition of ergosterol biosynthesis, and increase in reactive oxygen species (ROS). These mechanisms damage and induce membrane loss, interfering on the integrity and physiology of the microorganism and finally leading to cell death (Cox *et al.*, 2000; Carson *et al.*, 2006; Kalagatur *et al.*, 2018).

After achieving complete inhibition, increases in oil concentration kept the values at maximum. For this reason, the quadratic plateau model presented the best fit for the relationship between oil dosage and inhibition percentage (Figure 2). The minimum concentration for total inhibition of *L. theobromae* mycelial growth was 0.75%, but regression analysis estimated that a dose of 0.47% could also generate complete inhibition.

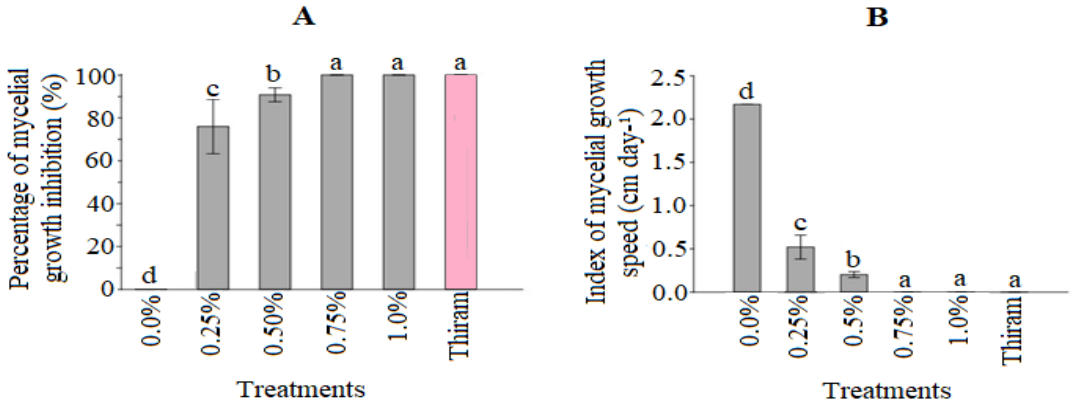
The inhibition percentages obtained with Melaleuca oil against *L. theobromae* differed from the inhibitions caused in other phytopathogenic fungi. Studies with the fungi *Aspergillus niger*, *Penicillium* sp., *Macrophomina phaseolina*, *Sclerotinia sclerotiorum*, *Alternaria alternata*, and *Colletotrichum gloeosporioides* have shown antifungal oil activity in concentrations ranging from 0.20 to 0.80% (Concha *et al.*, 1998; Martins *et al.*, 2010; Marinelli *et al.*, 2012; Ramos *et al.*, 2016). These differences may be due to the diversity of defense mechanisms of each fungal species and the selection of fungi resistant to compounds in the oil (Wardle and Parkinson, 1990; Takahashi and Melhem, 2014). As Melaleuca essential oil exerts different fungitoxic activities depending on the studied

microorganism, it generates different minimum concentrations for each species, opening opportunities for further investigated.

The essential oil from other plant species also significantly inhibited *L. theobromae*. For example, mint oil (*Mentha arvensis*) at a minimum concentration of 0.25% completely inhibited *L. theobromae* mycelial growth (Peixinho *et al.*, 2017a).

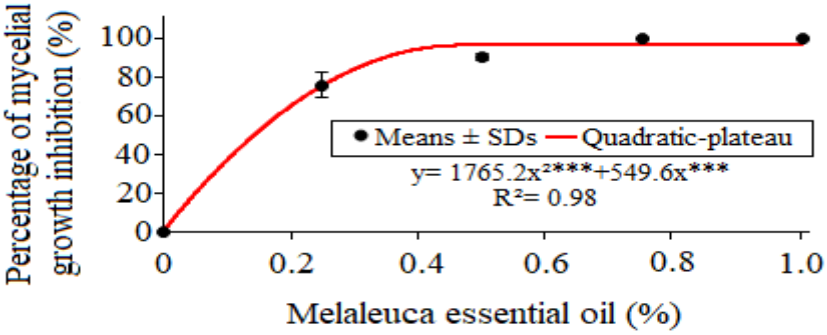
Citronella (*Cymbopogon nardus*) oil at 1.0% also inhibited the growth of this fungus by 100%, while clove oil (*Syzygium aromaticum*) at 1.5% achieved inhibition of only 35.6% (Peixinho *et al.*, 2017b).

Fig.1A Inhibition of mycelial growth of *Lasiodiplodia theobromae* in the different concentrations of Melaleuca essential oil and the control treatments. **1B.** Mycelial growth speed of *Lasiodiplodia theobromae*in the different concentrations of Melaleuca essential oil and the control treatments



Superscript treatment with the same letter were not significantly different from each other by the Mann-Whitney test ($p>0.05$)

Fig.2 Effect of different concentrations of Melaleuca essential oil on the mycelial growth of *Lasiodiplodia theobromae*.The red line shows the direction of effect estimated by plateau-quadratic regression



*** P < 0.001;

Fig.3 Effect of different concentrations of Melaleuca essential oil on the incidence of infected seeds by *Lasiodiplodia theobromae*

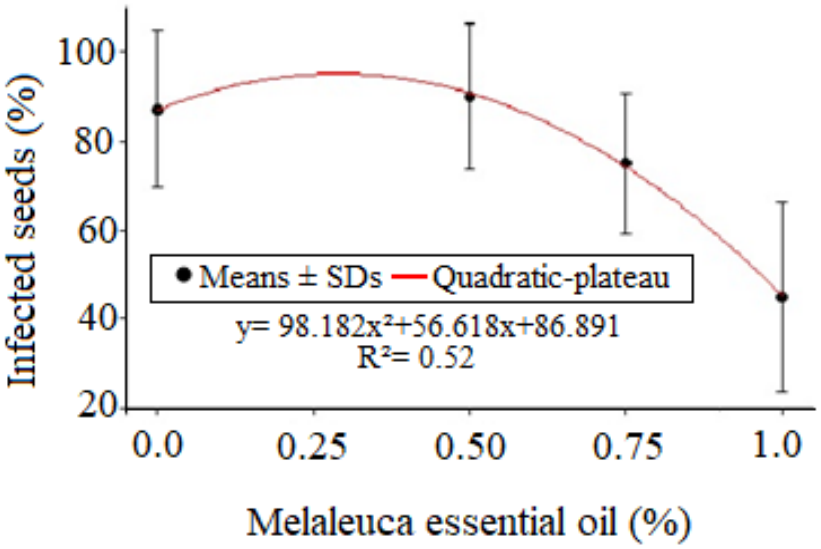
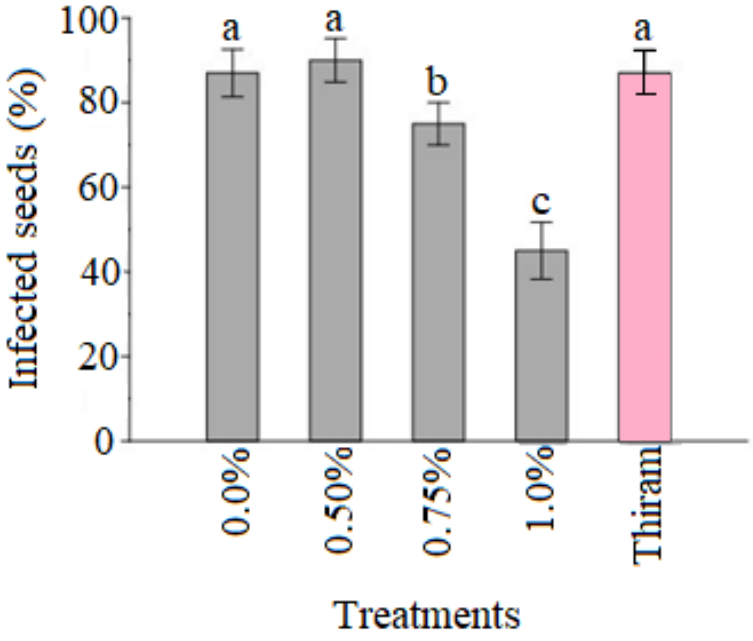


Fig.4 Percentage of infected seed by *Lasiodiplodia theobromae* after the treatment with the different concentrations of Melaleuca essential oil and the control treatments



Superscript treatment with the same letter were not significantly different from each other by the Mann-Whitney test ($p>0.05$)

The fungitoxic control promoted by the essential oils occurs through synergism or antagonism between several of its compounds, which act through several mechanisms on several targets at the same time (Hammer *et al.*, 2004; D´Auria *et al.*, 2001; Yu *et al.*, 2015). These characteristics confer advantages over the synthetic fungicide since they hinder the evolution of phytopathogen resistance (Feng and Zheng, 2007).

In vivo experiment (maize seeds)

The Melaleuca essential oil at 0.75 and 1% provided a reduction in the incidence of *L. theobromae* in maize seeds, differing significantly from the incidence in untreated seeds (negative control) and treatment with Thiram fungicide (positive control) (Figure 3 and4). None of the concentrations eradicated the pathogen, but the increase in oil concentration decreased the number of colonies (Figure 3). Applying the equation generated by the quadratic polynomial model, we estimated that the minimum dose to eradicate the fungus would be 1.26%.

Thus, our results demonstrate that biologically active compounds in Melaleuca oil promote a significant antifungal effect on *L. theobromae* mycelial growth under both *in vitro* and seed treatment conditions. By using the oil at a concentration of 0.75%, we obtained total inhibition of mycelial growth under *in vitro* conditions. However, seed treatment requires higher doses for effective fungus inhibition (less than 45% of infected seeds).

The efficiency of tea tree oil on *L. theobromae* observed in our *in vivo* tests differed from some results previously obtained in the fight against other fungi. For example, the treatment of soybean seeds with Melaleuca oil completely inhibited the incidence of *Aspergillus* spp. and *Phomopsis* sp. (Morais *et al.*, 2008). In bell pepper seeds, concentrations of 0.50% and 0.75% eradicated the fungus *Colletotrichum gloesporioides* (Nascimento, 2017). The divergence of the results shows that the effectiveness of the oil varies according to the pathogen species. Fungi mycelial growth rate (IMGS) may be one of the factors that interfere with control efficacy, an issue that deserves further studies (Amponsah *et al.*, 2012; Bester *et al.*, 2007; Rolshausen *et al.*, 2010).

The effect of 1.0% Melaleuca oil against *L. theobromae* in maize seeds was superior to the effect of Thiram synthetic fungicide, with an average incidence of 45% in contrast to 87% of Thiram (Figure 4). Despite the need for a higher oil concentration than the Thiram concentration to reduce the number of infected seeds, the use of a less harmful natural product may be a safer alternative for fungus control.

However, even if it is natural oil, the recommended minimum concentrations should be considered to avoid toxicity to humans and to minimize environmental impacts. We suggest the evaluation of each component of the oil isolated, as the action of different chemical compounds in high dosage may increase the oil toxicity.

Melaleuca essential oil completely inhibited *L. theobromae* growth under *in vitro* conditions, with a minimum dose of 0.75%. Also, the oil reduced the incidence of black rot in maize seeds, with an optimal effect from the 1% concentration, presenting greater efficacy than the commercial Thiram fungicide. Our results can be useful in formulating pesticides based on Melaleuca essential oil for use in agroecological crops, minimizing the impacts of synthetic pesticides.

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