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In vitro fungitoxic potential of *Lippia gracilis* (Schauer) essential oil against phytopathogens

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Abstract

This study evaluates the *in vitro* effects of *Lippia gracilis* essential oil on the mycelial growth of phytopathogenic fungi. Experiments were carried out using a completely randomized design to assess the effects of eight treatments. Five replicates were evaluated for each experimental group. The essential oil was incorporated into the potato dextrose culture medium and poured into Petri dishes. Treatments were comprised of different concentrations of the oil (0.0125, 0.025, 0.05, 0.1, and 0.2%), a negative control (0.0%), and two positive controls (commercial fungicides). The plates were inoculated with fungi including *Colletotrichum gloeosporioides*, *C. musae*, *C. fructicola*, *C. asianum*, *Alternaria alternata*, *A. brassicicola*, *Fusarium solani*, *F. oxysporum* f. sp. *cubense*, and *Lasiodiplodia theobromae* and were incubated for seven days at 27 ± 2°C. The following variables were measured to verify the differences observed among treatments: percentage of mycelial growth inhibition and index of mycelial growth speed. All concentrations of *L. gracilis* oil inhibited the mycelial growth of the fungal species evaluated. The complete inhibition was observed between concentrations of 0.0125 and 0.1%. Treatment with oil inhibited fungal growth with similar, or even greater, efficiency than commercial fungicides.. We recommend the development of *in vivo* tests to verify whether *L. gracilis* essential oil can protect against fungal disease in live plants.

Keywords: *Alternaria* spp., alternative control, *Colletotrichum* spp., *Fusarium* spp., *Lasiodiplodia* sp., phytopathogenic fungi, fungitoxicity.
Abbreviations: °C_degrees Celsius; cm day^{-1}_centimeters per day; df_degrees of freedom; g L^{-1}_grams per liter; EO_Essential oil; IMGS_Index of mycelial growth speed; MICest_Minimum estimated concentration; MIC_Minimum inhibitory concentration; mL L^{-1}_mililiter per liter; PGI_Percentage of mycelial growth inhibition; ppm_part per million; SD_standard deviation; µL L^{-1}_microliter per liter; µL mL^{-1}_microliter per milliliter.

Introduction

Agricultural production faces several phytosanitary problems worldwide, which negatively affect productivity and the quality of agricultural products. For example, diseases caused by phytopathogenic fungi result in production losses of a wide variety of crops and, subsequently, result in economic losses (Brum et al., 2014).

Fungal genera including *Colletotrichum, Alternaria*, *Fusarium*, and *Lasiodiplodia* are comprised of species capable of causing disease in a wide variety of economically important crops. Among these, we highlight the stem bleeding and gummosis, which are primary diseases of cashew trees (*Anacardium occidentale* L.), caused by *L. theobromae* (Muniz et al., 2011); Panama disease, which is a significant disease of banana (*Musa* sp.), caused by *F. oxysporum* f. sp. *cubense* (Castro et al., 2008); soybean (*Glycine max* L.) common bean (*Phaseolus vulgaris* L.), and cassava (*Manihot esculenta* Cranz.) root rot, caused by *F. solani* (Poltronieri et al., 2002; Aoki et al., 2003, Teixeira et al., 2012); anthracnose of papaya (*Carica papaya* L.), mango (*Mangifera indica* L.), and banana, caused by *Colletotrichum* spp. (Bonnet et al., 2013; Ribeiro et al., 2016; Shivas et al., 2016); the rot, which occurs in papaya and muskmelon (*Cucumis melo* L.), is caused by *A. alternata* (Nascimento et al., 2014; Barboza, 2015); and diseases of *Brassica* species caused by *A.*

brassicicola (Deep and Sharma, 2012).

The conventional treatment of fungal diseases involves the use of chemical pesticides that, due to their high toxicity, contaminate the environment (Candido e Silva and Melo, 2013), affect human health, and raise agricultural production costs (Perina, 2014). In addition, they limit the export of agricultural products, as a result of the restrictive norms of several countries with regard to the use and residues of chemicals for growing food (Adami et al., 2016).

To reduce hazards posed by use of agrochemicals, alternative products have been developed that are designed to reduce the use of conventional synthetic pesticides. An alternative product should effectively control phytopathogens, have a low cost, not produce environmental or human health risks. Among products widely tested for this purpose, the essential oils extracted of aromatic plants, whose antifungal activity has been previously reported in several studies (Carnelossi et al., 2009; Santos et al., 2010; Oliveira Júnior et al., 2013; Santos et al., 2014; Peixinho, Ribeiro and Amorim, 2017), are of particular interest.

Studies evaluating the essential oil (EO) of *Lippia gracilis* (Schauer) demonstrated that it possessed a diversity array of biological activities, which included antimicrobial effects. Chemical analysis of *L. gracilis* EO revealed that thymol and/or carvacrol were its primary components (Matos et al., 1999; Silva et al., 2008; Ferraz et al., 2013; Bitu et al., 2014; Franco et al., 2014), which have been shown to possess antimicrobial properties (Albuquerque et al., 2006; Kordali et al., 2008; Morcia et al., 2012; Abbaszadeh et al., 2014). In the control of phytopathogens, this EO inhibits the development of *Macrophomina phaseolina* (Ugulino et al., 2018*Alternaria* sp. (Barboza, 2015), and *Rhizoctonia solani* (Silva et al., 2012).

The use of *L. gracilis* EO against phytopathogens has several advantages over conventionally used, synthetic pesticides, for example, it rapidly degrades in the environment (Hu and Coats, 2008) and has a low degree of toxicity (Guilhon et al., 2011). This work aims to evaluate the *in vitro* effects of *L. gracilis* EO on the mycelial growth of a set of phytopathogenic fungi. Our results will inform agricultural practices aiming to promote use of EOs to enhance the management of phytopathogens, reducing negative impacts associated with the exclusive use of chemical pesticides.

Results and Discussion

Mycelial growth inhibition and growth rate

The *L. gracilis* EO reduced the mycelial growth and overall growth rates of all phytopathogens considered at all EO concentrations assessed (Figure 1). The percentage of inhibition increased significantly as EO concentration increased until maximum inhibitory values were reached (PGI = 100%; Figure 2).

The total inhibition of *C. gloeosporioides*, *C. musae*, *A. alternata*, *A. brassicicola*, *F. solani*, and *F. oxysporum* f. sp. *cubense* occurred at EO concentrations of 0.025% and greater. Further, growth of *C. fructicola* and *L. theobromae* was completely inhibited with 0.1% EO. All tested concentrations were capable of complete growth inhibition of *C. asianum*. The concentrations referred to here are the lowest concentrations capable of inhibiting 100% of the growth each organism tested and will subsequently be referred to as minimum inhibitory concentrations (MICs; Table 1).

Equations generated by regressions using the quadratic-plateau model allowed the calculation of minimum estimated concentrations (MICest), which were slightly different from MIC (Table 1). The estimations suggested that the mycelial growth of *C. gloeosporioides*, *C. musae*, *C. fructicola*, *F. solani*, and *L. theobromae* could be inhibited entirely at lower concentrations than the observed. Growth rate indices decreased as EO concentrations increased until the growth of phytopathogens was completely inhibited at their respective MICs (see table 1). These rates significantly differed from negative controls, which had the fastest rates of growth (Table 2).

Percentages of mycelial growth inhibition (PGIs) and mycelial growth rate indices (IMGS) differed significantly between the nine fungal species and six concentrations of the EO evaluated (Table 3). Also, sources of variation interacted significantly, as each fungal species reacted differently to increases in the concentrations of EOs. In all cases, dose-dependent behavior was observed, that is, inhibition increased as concentration increased. However, minimum inhibitory concentrations varied among species. This result suggests that the fungal species have different susceptibility profiles to *L. gracilis* EO, which may be a result of differences among the defense mechanisms employed by the species tested.

Our results demonstrate the powerful antifungal effect of *L. gracilis* EO, which inhibited the growth of evaluated fungi even in low concentrations. Although mechanism details of *L. gracilis* EO-mediated growth inhibition are poorly understood, it is believed that these compounds dissolve fungal membranes and cell walls by altering cell permeability, which promotes the loss of essential macromolecules (Isman and Machial, 2006; Pina-Vaz et al., 2004; Segvić Klarić et al., 2007). The fungicidal activity of thymol and carvacrol involves inhibition of ergosterol biosynthesis and causes severe damage to cell membranes, an action that leads to cell death (Ahmad et al., 2011).

Also, using *L. gracilis* EO at concentrations close to or greater than those evaluated in this work, other authors have obtained similar results. For example, under *in vitro* conditions, Ugulino et al. (2018) achieved total inhibition of *Macrophomina phaseolina* growth at all tested concentrations (0.4 to 1.0%). Further, when evaluating the growth of *Alternaria sp.*Barboza (2015) showed that growth of the species was completely inhibited when given a dose of 750 µL L^{-1} EO (0.075%). Albuquerque et al. (2006) reported the total inhibition of *Curvularia lunata* growth when 440 µL L^{-1} (0.044%) EO was provided.

Other species of the genus *Lippia* have the same fungitoxic potential as *L. gracilis* in the control of phytopathogens. Souza Júnior et al. (2009) assessed the effect of *L. sidoides* and *L. citriodora* EOs against *C. gloeosporioides* and obtained complete inhibition of fungal growth at all tested concentrations, which ranged from 1 to 10 µL mL^{-1} (0.1 to 1.0%). In the control of *L. theobromae*, Mota et al. (2002) reported that the *in vitro* effect of *L. sidoides* EO was the total inhibition of fungal growth at all concentrations tested, which ranged from 0.05 to 1.0%. The EOs obtained from different species of the genus *Lippia* have been shown to have similar chemical compositions, with only small variations only in the proportion of their components (Cruz et al., 2013). This compositional similarity may explain similarities reported with respect to the capacities of

these EOs to

Table 1. Minimum inhibitory concentrations of *Lippia gracilis* essential oil against a set of phytopathogens.

Phytopathogens	MIC* (%)	MICest** (%)
Colletotrichum gloeosporioides	0.025	0.023
Colletotrichum musae	0.025	0.023
Colletotrichum fructicola	0.1	0.048
Colletotrichum asianum	0.0125	0.041
Alternaria alternata	0.025	0.042
Alternaria brassicicola	0.025	0.029
Fusarium oxysporum f. sp. *cubense*	0.025	0.030
Fusarium solani	0.025	0.022
Lasiodiplodia theobromae	0.1	0.034

*Minimum inhibitory concentration observed on *in vitro* test; **Minimum inhibitory concentration estimated by the regression analysis using quadratic-plateau model.

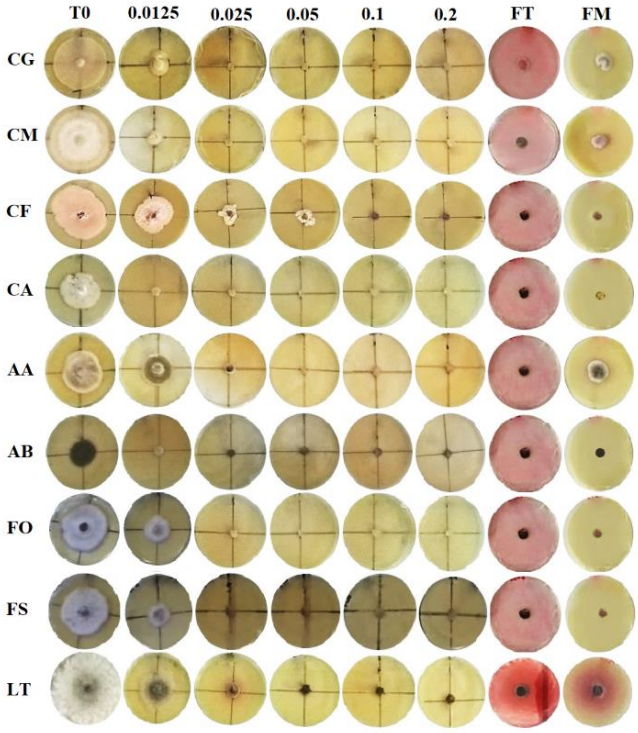

Fig 1. Comparison of the mycelial growth of phytopathogens post-exposure to treatment with *Lippia gracilis* essential oil and controls. The negative control treatment is indicated by T0, FT and FM indicate positive controls (1 ml L^{-1} of Thiram and 0.2 g L^{-1} of Mancozeb, respectively); 0.0125 to 0.2% are *L. gracilis* essential oil concentrations (%), CG indicates *Colletotrichum gloeosporioides*, CM indicates *Colletotrichum musae*, CF indicates *Colletotrichum fructicola*, CA indicates *Colletotrichum asianum*, AA indicates *Alternaria alternata*, AB indicates *Alternaria brassicicola*, FS indicates *Fusarium solani*, FO indicates *Fusarium oxysporum* f. sp. *cubense* and LT indicates *Lasiodiplodia theobromae*.

Table 2. Index of mycelial growth speed (cm day^{-1} ± SD) values of phytopathogenic fungi after treatment with minimum concentrations of *Lippia gracilis* essential oil and the control treatments.

Phytopathogen	Negative control	MIC*	Tiram	Mancozeb
Colletotrichum gloeosporioides	0.88 ± 0.01 a**	NG*** b	NG b	NG b
Colletotrichum musae	1.50 ± 0.07 a	NG b	NG b	0.08 ± 0.08 b
Colletotrichum fructicola	0.82 ± 0.23 a	NG b	NG b	NG b
Colletotrichum asianum	0.50 ± 0.05 a	NG b	NG b	NG b
Alternaria alternata	0.58 ± 0.04 a	NG b	NG b	0.13 ± 0.11 b
Alternaria brassicicola	0.33 ± 0.01 a	NG c	NG c	0.11 ± 0.02 b
Fusarium solani	0.89 ± 0.08 a	NG c	0.02 ± 0.02 c	0.24 ± 0.02 b
Fusarium oxysporum f. sp. *cubense*	0.81 ± 0.02 a	NG c	0.06 ± 0.02 b	NG c
Lasiodiplodia theobromae	2.56 ± 0.08 a	NG b	NG b	NG b

*Minimum inhibitory concentration observed on *in vitro* test (see values for each species in the Table 1). **Letters should be compared among columns; averages with the same letter have no statistically significant difference (Mann-Whitney test, $p > 0.05$). ***NG = No growth (IMGS = 0.00 ± 0.00 cm day^{-1}).

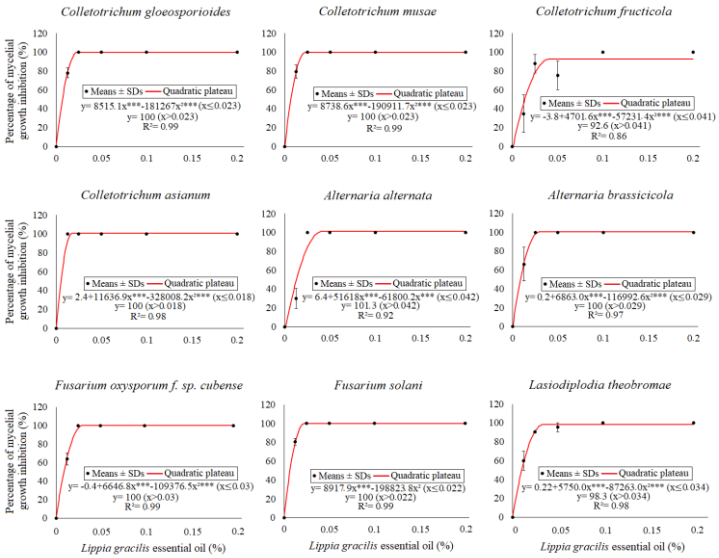

Fig 2. Effect of different concentrations of *Lippia gracilis* essential oil on the mycelial growth of phytopathogens. The red line shows the direction of effect estimated by a quadratic plateau regression. *** P < 0.001.

Table 3. Analysis of variance associated with the observed percentage of mycelial growth inhibition (PGI) and index of mycelial growth speed (IMGS) among eight treatments of nine fungal species.

Percentage of mycelial growth inhibition (PGI)					
Sources of variation	Sum of sqrs	Df	Mean square	F-value	P-value
Fungal species	5592.6	8	699.08	7.1956	0.0001
Treatments	3.82E+05	15	25444	261.89	0.0001
Interaction	12486	120	104.05	1.071	0.0001
Residual	20985	216	97.153		
Total	4.21E+05	359			
Index of mycelial growth speed (IMGS)					
Sources of variation	Sum of sqrs	Df	Mean square	F-value	P-value
Fungal species	5.3324	8	0.66655	51.643	0.0001
Treatments	36.269	15	2.4846	192.5	0.0001
Interaction	15.487	120	0.12906	9.9989	0.0001
Residual	2.7879	216	0.012907		
Total	60.876	359			

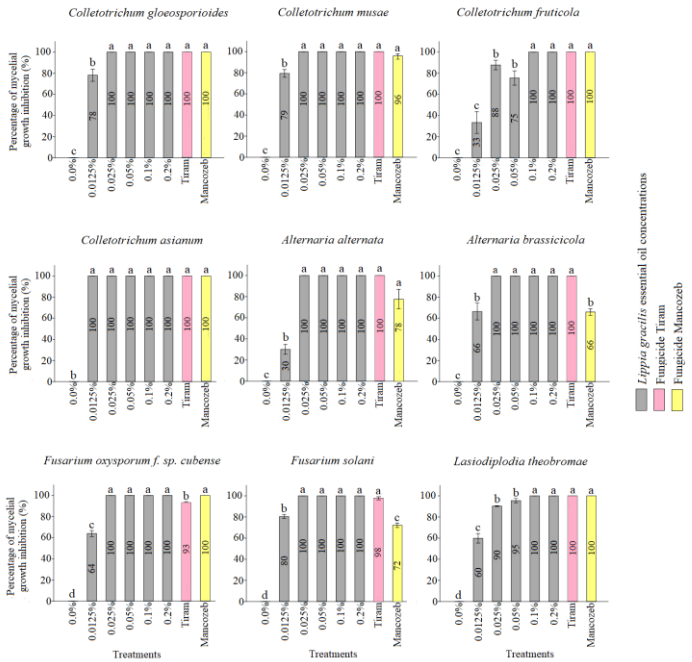

Fig 3. Inhibition of the mycelial growth of phytopathogens after they were treated with different concentrations of *Lippia gracilis*

essential oil and controls.

inhibit fungal growth. Many EOs extracted from other plant species have shown either similar, or inferior effects against the fungal species assessed. For example, the EO of Brazilian Peppertree (*Schinus terebinthifolius*) inhibited only 79% of the mycelial growth of *C. gloeosporioides* at a concentration of 0.5% (Oliveira Júnior et al., 2013). Further lemongrass EO (*Cymbopogon citratus*) at concentrations ranging from 0.15 to 1.25 µL mL^{-1} (0.015 to 0.125%) inhibited only 37.8 and 37% of the mycelial growth of *C. fructicola* and *C. asianum*, respectively (Oliveira et al., 2018).

The Mexican-tea EO (*Chenopodium ambrosioides*) completely inhibited the growth of *C. gloeosporioides*, *C. musae*, and *F. oxysporum* at 0.3% (Jardim et al., 2010). Lower concentrations resulted in the moderate inhibition of growth, which varied between 40 and 50%. Peppermint EO (*Mentha piperita*) completely inhibited the growth of the aforementioned fungi at a concentration of 0.2% or greater (Freire et al., 2012). Lemongrass EO completely inhibited *F. solani* at doses of 500, 1,000 and 1,500 ppm (0.05, 0.1, and 0.15%, respectively) (Mishra and Dubey, 1994). A concentration of 2% frankincense EO (*Acacia farnesiana*) inhibited *A. brassicicola* growth 71.29%, while a 1.0% concentration of acacia oil (*Boswellia carterii*) resulted in the complete inhibition of the species (Udomsilp et al., 2009). On the other hand, the inhibitory effects of peppermint EO on *A. alternata* growth ranged from 37.1 to 41.6% when 1.0% EO was assessed. Through the regression analysis, the estimated maximum concentration for total inhibition of growth was calculated to occur at a concentration of 2.26% EO (França et al., 2018).

Each EO tested was determined to possess antimicrobial activity against a range of microorganisms. However, the concentrations required to obtain significant effects vary depending on the pathogen subjected to treatment. Also, antifungal efficiency depends the susceptibility of the microbe assessed and the chemical composition of the EO, which varies between plant species (Antunes and Cavaco, 2010).

Comparison of L. gracilis EO and fungicides

We compared the fungitoxic effects of the EO and two synthetic fungicides (Tiram and Mancozeb) aiming to determine possible antifungal applications of *L. gracilis* EO with respect to their use combatting phytopathogens. Outcomes when using the EO were generally equal to or improved relative to treatment using the fungicides tested (Figure 3). Percentage of inhibition of the mycelial growth rates of *C. gloeosporioides*, *C. musae*, *C. fructicola*, *C. asianum*, *A. alternata*, and *L. theobromae* using the MIC of the EO were similar to the rates obtained with the commercial fungicides. In the cases of *A. brassicicola* and *F. solani*, the effects were superior to the fungicide Mancozeb and identical to the Tiram. Conversely, *F. oxysporum* f. sp. cubense, use of the EO produced results that were superior to Tiram and similar to Mancozeb. The similarity and even superiority of *L. gracilis* EO compared to commercial fungicides suggests that, under *in vitro* conditions, the EO could functionally completely replace the use of either of the products tested.

Due to their high degree of chemical complexity, EOs promote microbial control through synergistic and antagonistic effects that occur between several of their components (Bagamboula et al., 2004; Russo et al., 2013). This complexity produces multiple inhibitory mechanisms that act on several targets simultaneously (Abdel-Kader et al., 2011; Hoyos et al., 2012). Synthetic agrochemicals, on the other hand, may have a single target and mechanism of action (Bebber and Gurr, 2015). The multiple pathways used to combat fungal growth provide EOs a key advantage over synthetic fungicides, because their complexity protects against the acquisition of microbial resistance (Feng and Zheng, 2007).

In the present study, growth of most fungal species tested was paralyzed at concentrations ranging from 0.025 and 0.1%, and some inhibitory concentrations were obtained at significantly lower dosages (> 60%). From an economic standpoint, this is a benefit of using *L. gracilis* EO, since use of minimal volumes of EO provides robust mycelial inhibition, which results in the economically efficient and effective control of pathogens.

The rapid degradation of EOs in the environment (Hu and Coats, 2008) and its low degree of risk to the health of producers and final consumers are key benefits of using *L. gracilis* EOs as alternatives to conventional fungicides in the control phytopathogens. In *in vivo* conditions, the EO could be integrated with other pre and post-harvesting techniques, reducing environmental impacts associated with the exclusive use of synthetic agrochemicals.

Despite the promising findings of this study, we recommend that future studies take place, which assess the capacity of the EO to control phytopathogens *in vivo*, since the effects of the EO may differ from those observed under *in vitro* conditions. It is essential to evaluate the activity of the *L. gracilis* EO on plant species of commercial value and also establish safe concentrations of the product.

Materials and Methods

Location of experiments and origins of materials

The study was conducted at the Center for Agrifood Science and Technology (CCTA) of the Federal University of Campina Grande (UFCG), Campus of Pombal. The experiment was carried out in the Phytopathology laboratory of this institution, from February to March of 2018.

The phytopathogenic fungi collection was provided by Prof. Maria Menezes (Federal Rural University of Pernambuco-UFRPE), which contained strains used for *in vitro* experiments including *C. gloeosporioides* 3331, *C. musae* 3499, *C. fructicola* 3811, *C. asianum* 3772, *A. alternata* 0878, *A. brassicicola* 0036, *F. oxysporum* f. sp. *cubense* 2141, *F. solani* 3826 and *L. theobromae* 4534. The fungi were preserved in sterile distilled water by the Castellani method for further use (Castellani, 1967).

L. gracilis EO was obtained using the steam distillation technique, which was carried out in the Laboratory of Biology II of the Department of Biology of State University of Rio Grande do Norte (UERN), Campus of Mossoró-RN.

Treatments and experimental design

Experiments had two sources of variation, eight treatments (five oil concentrations, one negative control, two positive

controls) and nine fungal species. Experiments were performed using a completely randomized 8 x 9 factorial design, with five replicates each. The treatments included pure *L. gracilis* EO at concentrations of 0.0125, 0.025, 0.05, 0.1, and 0.2%; a negative control (culture medium without supplementation); and two positive controls (culture medium supplemented with Tiram and Mancozeb commercial fungicides at concentrations recommended by their manufacturers of 1 mL L^{-1} and 0.2 g L^{-1}, respectively). The concentrations used were selected based on work by Ugulino et al. (2018), that reported use of the oil between 0.4 to 1.0%. To obtain the final concentrations, we used the direct dilution procedure in a culture medium (Pereira et al., 2006).

Experimental procedures

Different treatments were incorporated into autoclaved and molten potato dextrose agar media. After cooling, the media was poured into Petri dishes 9 cm in diameter under aseptic conditions. After solidification, culture medium disks, 1 cm in diameter, containing mycelia of each fungal species were transferred to the center of each treatment-containing plate. The plates were then wrapped in plastic film and stored in a biochemical oxygen demand incubator at 27 ± 2°C.

We measured the growth of each colony daily one filled the entire surface of the culture medium for a maximum period of 7 d. The daily measurements consisted of the average of two perpendicular diameters, which were obtained with the aid of a graduated ruler. The mean diameters facilitated the calculation of percentage of mycelial growth inhibition (PGI; Bastos, 1997) was calculated according to formula (1) as follows:

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

And the and index of mycelial growth speed (IMGS; Oliveira, 1991) was calculated according to equation (2) as follows:

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

Statistical analysis

The effects of fungal species, oil concentrations, and interactions between these two variables on PGI and IMGS values were evaluated using a two-way PERMANOVA (ANOVA with 9,999 permutations). We used this non-parametric statistic due to the absence of variance associated with some treatments. Regressions were performed using quadratic plateau models to verify the effects of oil concentrations on fungal growth.

To evaluate differences between oil and fungicide (positive control) treatments, we applied Mann-Whitney pairwise comparisons. All differences with a probability value below 5% were considered significant. The analyses were performed using R 3.5.1 software (R Core Team, 2019) and the Past 3.12 program (Hammer et al., 2001).

Conclusion

Under *in vitro* conditions, *L. gracilis* (Schauer) EO completely inhibited mycelial growth of the fungal species *C. gloeosporioides*, *C. musae*, *C. fructicola*, *C. asianum*, *A. alternata*, *A. brassicicola*, *F. oxysporum* f. sp. *cubense*, *F. solani*, and *L. theobromae* when provided at concentrations of 0.0125 to 0.1%. The effect of the oil was similar or superior to synthetic fungicides Tiram and Mancozeb.

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