Control of *Alternaria alternata* using Melaleuca essential oil (*Melaleuca alternifolia*)

.

ABSTRACT

Aims: This study aimed to evaluate the fungitoxic potential of melaleuca essential oil on the mycelial growth of *Alternaria alternata* under *in vitro* condition and the treatment of cowpea beans.
Study desing: The experiments comprised completely randomized designs: Eleven treatments with five replicates on *in vitro* test; and six treatments with five replicates on *in vivo* test.
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, since February 2018 to February 2019.
Methodology: In the *in vitro* experiment, the essential oil was incorporated into the culture medium and poured into Petri dishes. The treatments consisted of different concentrations of the essential oil (0.0125, 0.025, 0.05, 0.1, 0.2, 0.25, 0.50, 0.75, and 1.0%), a negative control (0.0%), and a positive control (Thiram). Discs of culture medium with fungal mycelia were inoculated in the center of the plates and incubated for seven days at 27±2ºC. The percentage of mycelial growth inhibition (PGI) and the index of mycelial growth speed (IMGS) was calculated to verify the difference between treatments. In the i*n vivo* experiment, the bean seeds were treated with different concentrations of EO (0.0, 0.2, 0.5, 1.0, and 5.0%), a negative control (0.0%), and positive control (Thiram). Seeds were inoculated with colonies of the fungus for 48 hours, and after that, we performed the seed sanity test.
Results: Under *in vitro* conditions, all concentrations of melaleuca essential oil reduced the mycelial growth of *A. alternata.* The oil reached complete inhibition of fungal growth from 0.2% concentration and above. In the cowpea treatment, the essential oil had no significant control over the percentage of infected seeds.
Conclusion: The melaleuca essential oil had a fungitoxic effect on the *A. alternata* under *in vitro* conditions. However, using the adopted methodology, on the cowpea bean seed treatment, the essential oil did not reduce the incidence of *A. alternata*.

Keywords: Alternative control, Cowpea bean, Mycelial growth, Phytopathogenic fungi, Tea-tree essential oil, Seeds disease, Vigna unguiculata.

1. INTRODUCTION

Cowpea bean (*Vigna unguiculata* (L.) Walp), popularly known as the string bean or macaçar bean, is a source of protein and staple food for a large part of the population of the North and Northeast of Brazil, thus one of the most important crops in the country [1]. According to CONAB [2], Brazil occupies the third position in world bean production with a cultivation area of approximately one million hectares, with the North and Northeast regions accounting for about 90% of the cultivated area [3].

Cowpea cultivation has a very competitive production cost, a factor that has increased the farmers' interest in the crop. In addition, Brazilian production is of high quality, enabling the

product to have good acceptance in all members of its production chain [4]. However, diseases represent a limiting factor to income expansion.

Fungi are the main phytopathogens that cause economic losses in bean crop. When present in the seed, they can cause miscarriages, deformations and discoloration of the bark, which always leads to the reduction of seed germination potential and vigor, and when allocated in the field will result in low or no yielding uneven plant stands [5]. Diseases caused by fungi with the greatest economic impact on bean crop are caused by *Macrophomina phaseolina* [6], *Fusarium* spp. [7], *Rhizoctonia solani* [8], *Curvularia* spp., *Trichoderma* spp. [9], *Alternaria* spp. [10], *Aspergillus* sp. and *Penicillium* sp. [11].

Considering bean pathogen control practices, treatment with synthetic agrochemicals has been a conventionally used one. However, the use of these products has been associated with significant damage to human health and the environment due to their high toxicity [12,13] besides favoring the emergence of resistant strains [14].

In this scenario, it is necessary to use alternative products to chemical pesticides that have similar efficacy but are not harmful to human health and the environment. Among the products studied are essential oils extracted from aromatic plants, which have fungitoxic properties on phytopathogens [15,16,17].

Melaleuca EO (*Melaleuca alternifolia*) has been studied for some years and its antimicrobial activity has been well documented. The main components of this oil are: terpinen-4-ol, cineol, terpenene, cymene, limonene and sabinene [18]. Most compounds have inhibitory activity against microorganisms [19], Terpinen-4-ol being the main constituent with antifungal activity [20]. In the control of phytopathogens its use has shown promising results in the control of fungi *Cercospora beticola* [21], *Aspergillus niger*, *M. phaseolina*, *Penicillium* sp. and *Sclerotinia sclerotiorum* [22], demonstrating a strong antimicrobial activity.

The antifungal action of essential oils is related to their ability to dissolve in lipid media, causing modifications in the cell membrane structure [23]. Due to their low toxicity and rapid degradation in the environment, the use of essential oils to combat phytopathogens may be a good alternative to synthetic pesticides [24]. Thus, this work aimed to evaluate the fungitoxic potential of melaleuca essential oil on the mycelial growth of *Alternaria alternata* under *in vitro* conditions and in the treatment of cowpea seeds.

2. MATERIAL AND METHODS

2.1 Place of experiments

The work was conducted at the Center of Science and Technology Agrifood (CCTA) of the Federal University of Campina Grande (UFCG), Campus of Pombal. The experiments were carried out in the Phytopathology laboratory, between February 2018 and February 2019.

2.2 Sampling

The fungal strain used was *Alternaria alternata* 0878, which was provided by the collection of phytopathogenic fungi Prof. Maria Menezes of the Federal Rural University of Pernambuco (UFRPE). The fungi were preserved in sterile distilled water by the Castellani method until the assay [25].

The pure essential oil of Melaleuca (*Melaleuca alternifolia*) was purchased at a local store specialized in natural products. The cowpea bean seeds (*Vigna unguiculata* L. Walp) were purchased at a commercial house in the city of Patos, Paraíba.

2.3 Screening of the antifungal activity of Melaleuca essential oil in vitro

Eleven treatments were used, 9 oil concentrations (0.0125, 0.025, 0.1, 0.2, 0.5, 0.25, 0.50, 0.75 and 1.0%), a negative control (without essential oil supplementation=0.0%) and a positive control (supplemented with 1 mL L^{-1} of the fungicide Thiram, which is the dosage indicated by the manufacture's). Five replicates of each treatment were arranged in completely randomized design (CRD).

The treatments were incorporated into PDA (Potato Dextrose Agar) culture medium just before pouring in sterilized Petri dishes. After solidification, one-centimeter mycelial disks were taken from the margins of 7days old culture and transferred to the center of each plate containing the treatments. The plates were then wrapped in plastic film and incubated in a BOD (Biochemical Oxygen Demand) at a temperature of 27±2°C.

The concentrations were chosen from an initial concentration based on the literature [26,27] and then gradually reduced until the addition of oil to the medium was no longer able to prevent the fungal growth. To obtain the final concentrations, the direct dilution procedure in a culture medium [28] was used.

Colony growth was measured daily until the colony took the entire surface of the culture medium in one of the plates or in a maximum period of 7 days. Mycelial growth evaluation consisted of daily measurements of the diameter of the colonies obtained through the average of two perpendicular measurements, using a digital caliper, resulting in the average daily growth for each repetition of each treatment.

The percentage of mycelial growth inhibition (PGI; [29]) and mycelial growth rate index (IMGS; [30]) were calculated according to formulas (1) and (2):

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

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

The minimum inhibitory concentration was considered the lowest oil concentration capable of totally inhibiting *Alternaria alternata* mycelial growth.

2.4 Screening of the antifungal activity of Melaleuca essential oil *in vivo* (on cowpea bean seeds)

The experiment consisted of a completely randomized design. The treatments consisted of sterilized distilled water solutions supplemented with 4 oil concentrations (0.2, 0.5, 1.0 and 5.0%), a negative control (without essential oil supplementation=0.0%) and a positive control (supplemented with 1 ml L^{-1} of the fungicide Thiram, which is the dosage indicated by the manufacture's). The concentrations used were determined based on the in vitro test. To emulsify the oin in the water Tween 80 (1 mL L-^{1}) was used [31].

The cowpea bean seeds were disinfected in 2.0% sodium hypoclorite solution for five minutes, washed with sterile distilled water twice and dried at room temperature. Afterwards they were immersed for five minutes in different solutions (treatments). After drying at room temperature, the artificial inoculation was performed.

The inoculation was done depositing the seeds on 7 days colonies of *A. alternata*. The seeds and the fungal colonies stayed for 48 hours in a BOD 27±2°C, with a 12-hour photoperiod [32].

After the treatment and inoculation, the samples were submitted to the sanity test, which was performed by the filter paper method with freezing [33]. Six hundred of cowpea bean seeds (100 per treatment) were used, distributed in Petri dishes (Ø=14 cm). In this method, ten seeds were placed at equal distances on each plate on triple layer of filter paper previously moistened in sterile distilled water and incubated initially for 24 hours on BOD at 27±2°C, with a 12-hour photoperiod. After this period, they were subjected to freezing (-20°C) for 24 hours, and then returned to the incubator for another five days.

After incubation, the seed were examined individually, using a stereoscopic microscope, for the quantification of seeds infected by *Alternaria alternata*. The results were expressed as percentage of infected seeds.

2.5 Statistical analysis

The effect of oil concentration on fungal growth was analyzed using regressions in quadratic plateau model for *in vitro* experiment and in linear model for *in vivo* experiment.

To test the difference between treatments with the essential oil and the treatment containing the fungicide (positive control), Mann-Whitney (Tukey nonparametric) multiple comparisons were applied. Non-parametric tests were used because of the lack of variance in the results of some treatments. Differences with a probability values below 5% were considered significant. The regressions were performed in the program R CoreTeam 3.5.1[34].

3. RESULTS AND DISCUSSION

3.1 *In vitro* antifungal assay

3.1.1 Effects of Melaleuca essential oil on *Alternaria alternata*

All tested concentrations of melaleuca essential oil reduced the mycelial growth of *Alternaria alternate* (Fig 1). The inhibition percentages increased significantly with the concentrations (P<.001) reaching the maximum value (PGI=100%) the 0.2% concentration of the oil (Fig 2A), which is the minimum inhibitory concentration (MIC). On the other hand, applying the regression equation in a quadratic plateau model, the estimated minimum concentration (MCest) was 0.33%.

The mycelial growth rate is a variable inversely proportional to the inhibition percentage. For this reason, it presented opposite behavior, with significant reduction with the tested oil concentrations (P<.001). The mycelial growth rate was more effectively reduced from the 0.2% concentration, in which growth paralyzed (IMGS=0.00 cm day^{-1}) (Fig 2B), differing from the negative control, which presented the highest growth speed (0.63 cm day^{-1}).

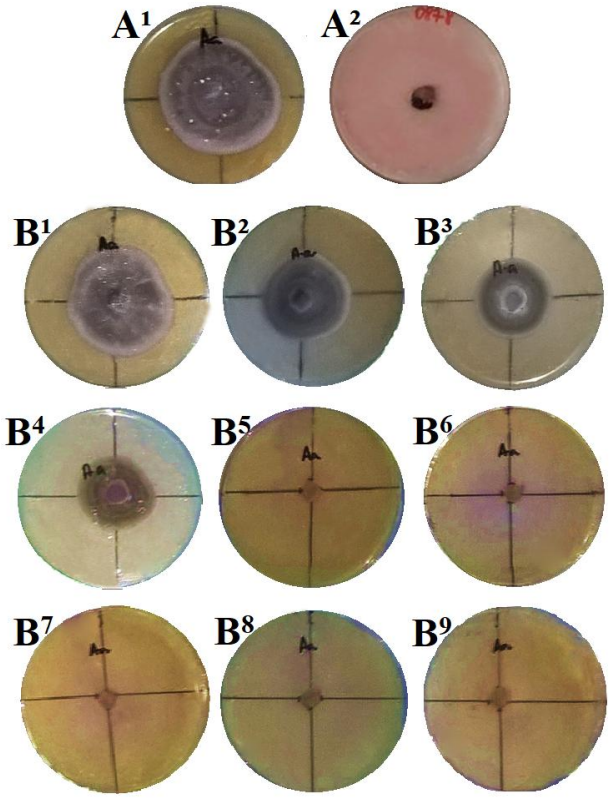

Fig 1. Comparation of *Alternaria alternate* mycelial growth in different treatments (melaleuca essential oil and the control treatments).

A^1 and A^2: Negative control (without essential oil supplementation) and positive control (1 mL L^{-1} of Thiram);

B^1, B^2, B^3, B^4, B^5, B^6,B^7,B^8 and B^9: Melaleuca essential oil at 0.0125, 0.025, 0.05, 0.1, 0.2, 0.25, 0.5, 0.75 and 1.0%, respectively.

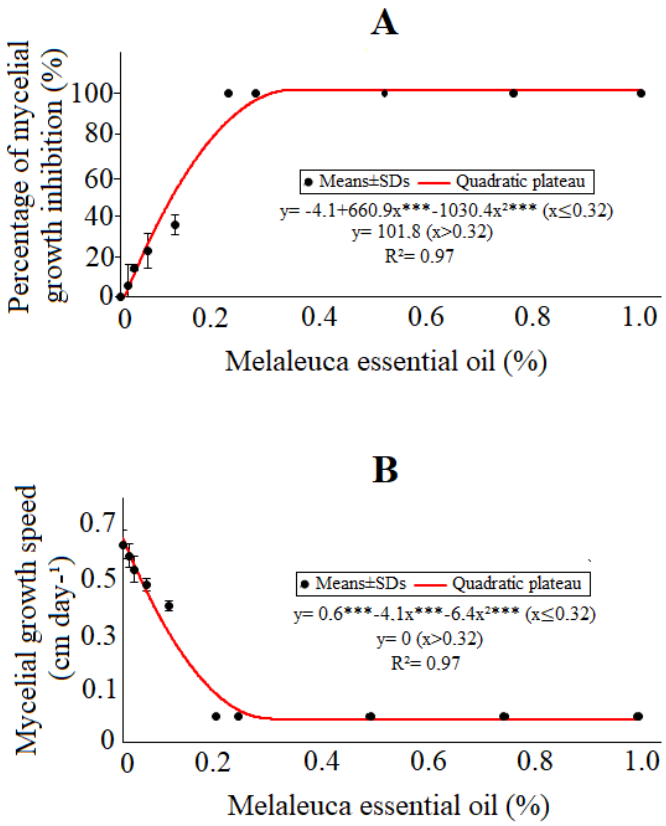

Fig 2. Inhibition percentage and mycelial growth speed of melaleuca essential oil against *Alternaria alternata*.

***Level of significance below 0.1% (*P*<.001)

Accordin to the literature, terpinen-4-ol is the major constituent of the melaleuca essential oil, which is associated with your high fungitoxic potential [35]. One of the antifungal mechanisms of action of melaleuca essential oil is the change in the permeability and fluidity of the cell membranes of the microorganisms. As these organisms are permeable to oil, the main effects found are inhibition of cell respiration and alteration in membrane structure and integrity, as well as leakage of essential intracellular materials. These events cause growth inhibition or even cell death [36,37].

Using tea tree oil at concentrations close to or greater than ours, other authors obtained similar inhibition results. For example, Martins et al. [22] obtained total inhibition of *M. phaseolina* and *S. sclerotiorum* at concentration 0.2% While in the control of *Alternaria radicina* and *A. dauci*, RIcioni and Orzali [38] reached the maximum inhibition from the concentration 0.5%.

Usion the essential oil of the other plant species on control of *A. alternata*, other authors obtained similar results as superior or inferior to ours. For example, the total inhibition was achieved by Chutia et al. [39], Guimarães et al. [40] and Barboza [41] using mandarin orange (*Citrus reticulata*), lemongrass (*Cymbopogon citratus*) and alecrim-da-chapada (*Lippia gracilis*) essential oil at concentrations of 0.2 mL/100mL (0.2%), 14.49 µg mL^{-1} (0.0014%) e 750 µL L^{-1} (0.075%), respectively. On the other hand, using peppermint essential oil (*Mentha piperita*), França et al. [42] obtained a maximum inhibition of 41.6% at a concentration of 0.8%. Thus, both the fungitoxic potential of essential oils on *A. alternata*,

as well as their minimum inhibitory concentrations will vary depending on the plant species from which the essential oil was extracted [43]. In addition, increasing inhibitory power as a function of increased concentration can either potentiate the effect or generate product waste.

To understand the potential of melaleuca essential oil as a fungicide on *A. alternata*, the fungitoxic effects of the essential oil and the fungicide Thiram (commercial synthetic fungicide) were compared. The essential oil, from the concentration of 0.2%, and the fungicide had similar inhibitions (Fig 3). This result suggests that under *in vitro* conditions the oil could replace the use of this agrochemical.

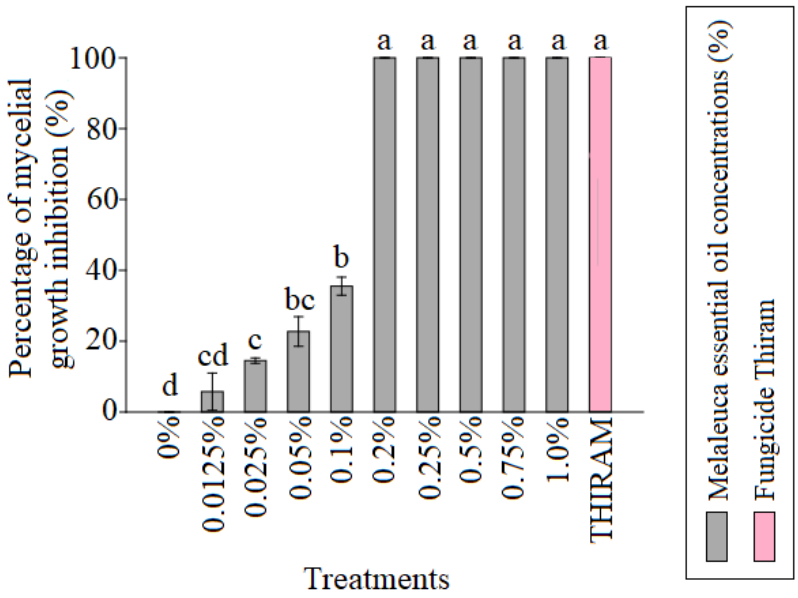

Fig 3. Effect of different treatments (melaleuca essential oil and the control treatments) on the mycelial growth inhibition of *Alternaria alternata*.
Superscript concentrations with the same letter were not significantly different from each other by the MannWhitney test (*P*>.05)

Due to the chemical complexity, the antifungal control promoted by essential oils is associated with their different constituents [44] through different mechanisms of action that act simultaneously on different targets [15]. These peculiar characteristics guarantee the advantage over synthetic fungicide, since they reduce the possibility of resistance by phytopathogens [45].

3.2 *In vivo antifungal assay*

3.2.1 Effects of Melaleuca essential oil on cowpea beans seed infected with *Alternaria alternata*

Contrary to the *in vitro* test, the melaleuca essential oil was impotent against the incidence of *A. alternata* in cowpea seeds (*P*<.001; Fig 4). The incidence of infected seeds at concentrations from 0.2 to 1.0% was similar to the negative control (*P*>.05), while at 5% concentration the incidence of phytopathogen increased substantially (Fig 5). On the other hand, the Thiram fungicide prevented the development of phytopathogen in the seeds.

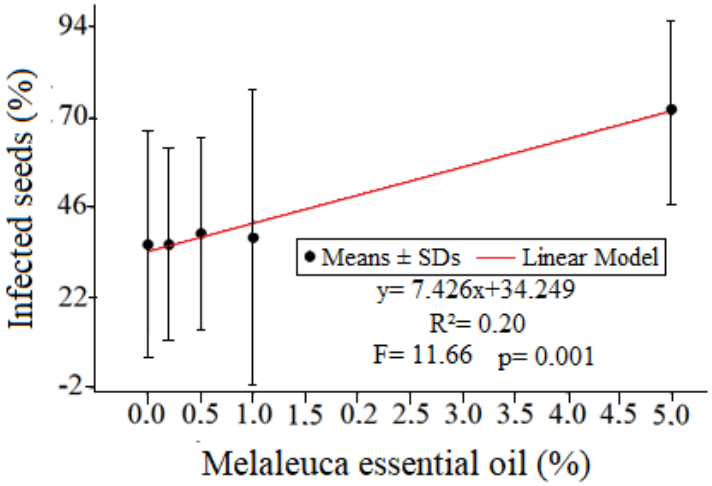

Fig 4. Effect of concentrations of melaleuca essential oil in the incidence of infected cowpea bean seeds by *Alternaria alternata*.

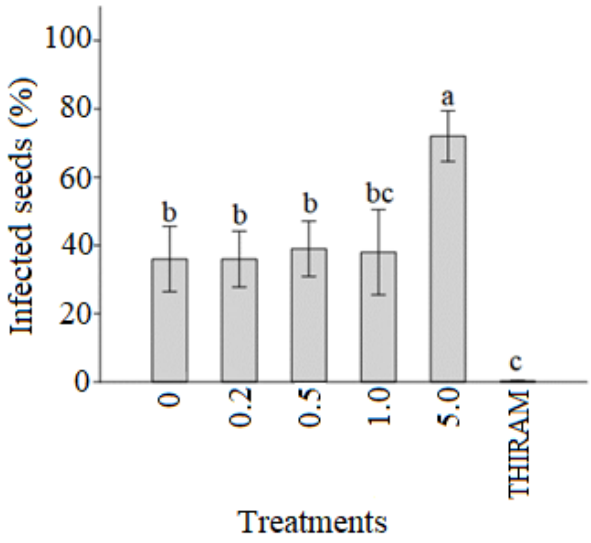

Fig 5. Percentage of infected cowpea bean seeds by *Alternaria alternata* after the treatment with different concentrations of melaleuca essential oil and the control treatments.

The potentiation of fungal growth in seeds treated with the melaleuca essential oil at 5% concentration may have occurred because of your low adherence to the surface of the seeds due to the high volatility of its constituents. Thus, during the incubation period some constituents may have evaporated and reduced to their antimicrobial capacity. The volatilization of oil constituents as well as their instability in the presence of light, heat and humidity, modify the atmosphere inside the Petri dishes, leading to the loss of the effectiveness of an oil that, under other conditions, inhibited fungal growth [46, 47].

Another hypothesis would be that some innocuous constituents present in higher concentrations became nutritive resources to the fungus, favoring its development even in the presence of fungitoxic components. Or, the high concentration of the essential oil may have affected the surface of the seeds and facilitated their colonization by the pathogen.

Despite our results, the use of other essential oil against phytopathogens in the bean seeds was successful. For example, the incidence of *Aspergillus* sp. and *Penicillium* sp. in beans treated with lemongrass (*Cymbopogon flexuosus* and *C. citratus*) and melaleuca (*Melaleuca* sp.) was reduced [48]. Also, citronella (*Cymbopogon* sp.), anise (*Pimpinella anisum*), and basil (*Ocimum basilicum*) essential oils at 1.5% concentration inhibited *Callosobruchus maculatus* [49].

Finally, despite the ineffectiveness of melaleuca essential oil in the treatment of cowpea been seed, this oil may be useful in the treatment of other seeds and other pathogens. Essential oils present a low risk to the environment, producers and consumers, and hinder the development of pathogen resistance [50]. Thus, further studies on the use of these oils in the management of plant pathogens are needed to make them a viable alternative for farmers.

4. CONCLUSIONS

Under *in vitro* conditions, melaleuca essential oil (*Melaleuca alternifolia*) totally inhibited the mycelial growth of *Alternaria alternata* from 0.2%, had a similar effect to the commercial fungicide Thiram. On the cowpea bean seed treatment, the essential oil had was not able to reduce the incidence of *A. alternata* using the adopted methodology.

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