Original Research Article

Control of phytopatogenic fungi using clove essential oil (*Syzygium aromaticum* L.)

ABSTRACT

Aims: This study evaluates the inhibitory potential of the clove essential oil (*Syzygium aromaticum* L.) on phytopathogenic fungi *in vitro* and on maize seeds.
Study design: The experiments comprised completely randomized designs: Seven treatments with five replicates on *in vitro* test; and four treatments with five replicates each, 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, from April to May 2018.
Methodology: In the *in vitro* test, the essential oil was incorporated into the PDA (Potato-Dextrose-Agar) culture medium and poured into Petri dishes. The treatments comprised different concentrations of the oil (0.0125, 0.025, 0.05, 0.1, and 0.2%), a negative control (0.0%), and a positive control (Tiram). Plates were inoculated with the fungi *Fusarium verticillioides*, *Macrophomina phaseolina*, and *Macrophomina pseudophaseolina* and incubated for seven days at 27±2°C. The percentage of mycelial growth inhibition (PGI) and mycelial growth rate index (MGRI) were estimated. In the *in vivo* test, maize seeds (AG1051 hybrid) were treated with the essential oil on concentrations equal or superior to the minimum inhibitory concentration found in the *in vitro* test, besides the negative and positive controls. The artificial inoculation was carried out in fungi colonies for 32 hours and the seed sanity test was performed. The percentage of seeds infected by the fungus was evaluated after seven days.
Results: *In vitro* conditions, clove oil totally inhibited the mycelial growth of *F. verticillioides*, *M. phaseolina* and *M. pseudophaseolina* at concentrations of 0.05, 0.1 and 0.1%, respectively. At 0.2% concentration significantly reduced the incidence of colonies of fungi *M. phaseolina* and *M. pseudophaseolina* in hybrid corn seeds AG 1051.
Conclusion: The clove essential oil had a fungitoxic effect on the phytopathogens evaluated, under *in vitro* and in the treatment of maize seeds.

Keywords: alternative control, fungitoxic effect, mycelial growth, plant diseases, Zea mays.

1. INTRODUCTION

Corn (*Zea mays* L.) is a cereal widely used in human and animal food, cultivated and consumed practically all over the planet [1]. In addition, it offers versatility of use in production systems, with a wide range of uses, from human and animal consumption *in natura* to the production of by-products by large industries in several areas, such as: pharmaceuticals, chemicals, beverages, fuel and which renders the grain into margarine, starch, flour, corn meal, bran, oil, glucose syrup and flakes for breakfast cereals [2,3,4].

Brazil is the third largest producer and exporter of maize according to the 11th USDA survey for the world corn crop 2018/2019, accounting for 94.5 million tons of world production, behind only to the United States and China, with production values of 366.3 and 257.3 million tons, respectively. Brazil's maize export volume increased 14.2% between 2017/18 and 2018/19, with export expectations of 29.0 million tons, behind only the United States and Argentina, with export values of 60.3 and 30.0 million tons, respectively [5].

Corn seeds are susceptible to several phytopathogens that cause damage during the production stages, especially in the establishment period of the crop. The presence of pathogens causes the weakening of seedlings and reduces the population of plants during the initial stages of development [6]. Many seed-borne diseases are caused by fungi, for example: stem rot and pink ear rot are caused by *Fusarium verticillioides* [7] and dry rot by *Macrophomina phaseolina* and *Macrophomina pseudophaseolina* [8,9].

In addition, seeds can act as vectors, transmitting disease from contaminated plantations to areas previously free from the pathogen [10]. These fungi survive on crop residues and on seeds in the form of mycelia, conidia and sclerotia, which later cause disease in the crop [11]. In Brazil, 100% of maize seeds are treated with fungicides and 85% with insecticides, and the use of high-quality seeds is also recommended, reducing the incidence and spread of pathogens both in the field and in storage [12,13].

In the field, phytosanitary problems are minimized through the conventional system of agricultural production [14]. This model of agriculture is based on the use of highly toxic chemical fertilizers and pesticides that cause a series of environmental damages such as the accumulation of harmful substances in soil and water, causing biological and ecological imbalances [15], as well as several health problems human [16]. The indiscriminate use of these products can also favor the emergence of resistant pathogens, requiring the progressive application of stronger agrochemicals that will cause even more significant damages [17].

In this context, many researchers have been engaged in the search for natural products that present in the composition substances with fungitoxic properties and that can be applied in the control of pathogens harmful to crops, that are less aggressive to human health and the environment. Among the natural products with these characteristics are the essential oils, which are complex compounds generated from secondary plant metabolites [18]. Several of these oils have low toxicity to humans and can be used with relative safety [19,20].

The essential oil of clove (*Syzygium aromaticum* L.) has been studied some years and your biological activities, including antifungal activity, have been documented on literature. Its main constituents are eugenol (70-90%), eugenol acetate (5-15%) and β-caryophyllene (up to 2.1%) [21, 22]. In the control of phytopathogens, their use presented promising results in low concentrations, in the control of *Rhizoctonia solani* [23] and *Fusarium* spp. [24].

The use of essential oils can reduce the need for the application of chemical pesticides, generating benefits for the environment and for the health of producers and consumers of agricultural products. This work aims to evaluate the fungitoxic potential of clove essential oil on the mycelial growth of fungi *Fusarium verticillioides*, *Macrophomina phaseolina* and *Macrophomina pseudophaseolina*, as well as the efficiency of this oil in the maintenance of sanitary quality of maize seeds.

2. MATERIAL AND METHODS

2.1 Place of experiments and obtaining materials

The experiments were conducted in the Laboratory of Phytopatology at the Center of Science and Technology Agrifood (CCTA) of the Federal University of Campina Grande (UFCG), between April and May of 2018.

We used the strains 3434 of *Fusarium verticillioides*, 2726 of *Macrophomina phaseolina* and 2709 of *Macrophomina pseudophaseolina* provided by the collection of phytopathogenic fungi Prof. Maria Menezes of the Federal Rural University of Pernambuco (UFRPE). The fungi were preserved in distilled water by the Castellani method until the assay [25].

The pure essential oil of clove (*Syzygium aromaticum* L.) was purchased at a local store specialized in natural products. Hybrid corn seeds AG1051 were purchased at a commercial house in the city of Pombal, with a minimum purity of 98% and a minimum germination of 85%.

2.2 Conduction of the Experiments

2.2.1 Experiment I: Effect of clove essential oil (*Syzygium aromaticum* L.) on *Fusarium verticillioides* and *Macrophomina* spp. *in vitro*

The experiments comprised completely randomized desing with seven treatments (5 oil concentrations, 1 negative control and 1 positive control) in five replicates each. The treatments consisted of autoclaved culture medium supplemented with the pure clove essential oil at different concentrations (0.0125, 0.025, 0.05, 0.1 and 0.2%); the negative control (without essential oil supplementation=0.0%) and the positive control (supplemented with 1 ml L^{-1} of the fungicide Tiram, which is the dosage indicated by the manufacture's).

The concentrations were chosen from an initial concentration based on the literature [26] 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, we used the direct dilution procedure in a culture medium [27].

The different treatments were incorporated into the autoclaved flux-PDA (Potato Dextrose Agar) culture medium. After cooling, the medium was poured into Petri dishes with 7.5 cm in diameter under aseptic conditions. After solidification, culture medium disks with 1 cm diameter containing mycelia of the fungus were transferred to the center of each plate containing the treatments. The plates were then wrapped in plastic film and incubated in a BOD type oven (Biochemical Oxygen Demand) at a temperature of 27±2°C.

Colony growth was measured daily until the colony took the entire surface of the culture medium in one of the plates or within a maximum period of 7 days. The evaluation of the mycelial growth consisted of daily measurements of the diameter of the colonies obtained through the average of two perpendicular measurements, using digital caliper, resulting in the average daily growth for each repetition of each treatment. With the result of the measures, the percentage of mycelial growth inhibition (PGI; [28]) and the index of mycelial growth speed (IMGS; [29]) were calculated according to the formulas (1) and (2):

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

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

2.2.2 Experiment II: Effect of clove essential oil (*Syzygium aromaticum* L.) on *Fusarium verticillioides* and *Macrophomina* spp. on maize seeds

The experiments comprised completely randomized designs. The treatments consisted of sterilized distilled water solutions supplemented with different concentrations of clove essential oil; a negative control (without essential oil supplementation=0.0%); and a positive control (supplemented with 1 ml L^{-1} of the fungicide Tiram, which is the dosage indicated by the manufacture's).

For *F. verticillioides*, 5 treatments were applied: The clove essential oil on concentrations 0.05, 0.1 and 0.2%; a negative control; and a positive control. For *M. phaseolina* and *M. pseudophaseolina*, 4 treatments were applied: The clove essential oil on concentrations 0.1 and 0.2; a negative control; and a positive control. The concentrations used were defined based on the *in vitro* test results. To allow the emulsion between oil and water we used Tween 80 (1 mL L^{-1}) [30].

The seed were disinfected in 2.0% sodium hypochlorite 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 corn seeds on the colonies of *F. verticillioides*, *M. phaseolina* and *M. pseudophaseolina* with 7 days of age. The contact time between seeds and fungal colonies was 32 hours on B.O.D (Biochemical Oxygen Demand) at 27±2 °C, with a 12-hour photoperiod [6].

After the treatment and inoculation, the samples were submitted to the sanity test, which was performed by the filter paper method with freezing [31]. One hundred seed were used per treatment, distributed on Petri dishes of 14 cm in diameter.

Ten seeds were placed equidistantly on each plate on triple layer of filter paper previously moistened in sterile distilled water and incubated initially for 24 hours on B.O.D (Biochemical Oxygen Demand) 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 evaluated individually, using a stereoscopic microscope, for the quantification of seeds infected by *Fusarium verticillioides*, *Macrophomina phaseolina* and *M. pseudophaseolina*, through the morphological characteristics of their structures. The results were expressed as percentage of seed infected by each fungus.

2.3 Statistical analysis

To verify the effect of the different concentrations of the essential oil on the growth of fungi, we used quadratic-plateau regressions model on the *in vitro* experiment data. The regressions were performed in the program R Core Team 3.5.1 [32].

To test the difference between treatments with the essential oil and the treatment containing the fungicide on the *in vitro* and *in vivo* tests Mann-Whitney (Tukey non-parametric) multiple

comparison was 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. These analyses were performed using Past 3.12 program [33].

3. RESULTS AND DISCUSSION

3.1 Experiment I: Effect of clove essential oil (*Syzygium aromaticum* L.) on *Fusarium verticillioides* and *Macrophomina* spp. *in vitro*

All tested concentration of clove essential oil reduced the mycelial growth and growth rate of evaluated phytopathogens. The inhibition percentages increased significantly with the concentrations tested until reaching and maintaining the maximum value (PGI=100%) at the higher concentrations (Figure 1).

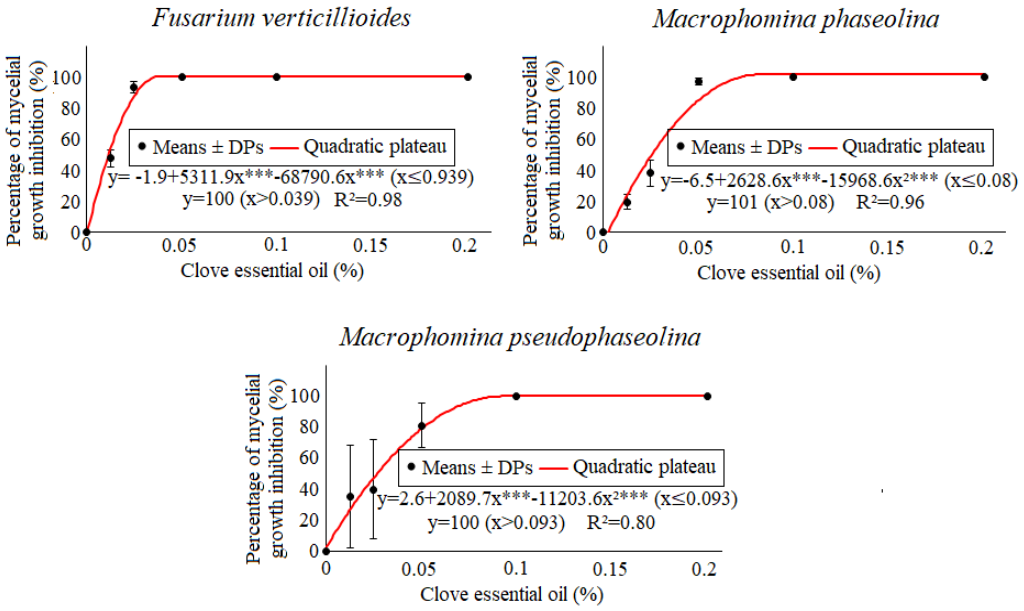

Fig 1. Effect of different concentrations of clove essential oil on the mycelial growth of phytopatogens.

****P<0.001*

The total inhibition of mycelial growth *Fusarium verticillioides* was obtained from the concentration of 0.05%, while the growth of *Macrophomina phaseolina* and *M. pseudophaseolina* was precluded with 0.1% of the oil. These were the lowest concentrations tested capable of totally inhibiting the growth of the evaluated phytopathogens. Below, we will refer to them as observed minimum concentrations (MCobs; Table 1).

Table 1. Minimum concentration of clove essential oil against different phytopatogens.

Phytopatogens	MCobs[1]	MCest[2]
Fusarium verticillioides	0,05	0,039
Macrophomina phaseolina	0,1	0,082
Macrophomina pseudophaseolina	0,1	0,093

[1]Minimum concentration determined on in vitro test;

[2]Minimum concentration estimated by the regression analysis on quadratic-plateau model.

On the other hand, using the equations generated by the regressions with the quadratic-plateau model, the estimated minimum concentrations (MCest; Table 1) were obtained, which were slightly different from MCobs. The estimate suggested that total inhibition of mycelial growth could be achieved applying lower concentrations than MCobs.

A decrease in the growth rate was observed with the essential oil concentration increased until the growth of phytopathogens was stopped when submitted to their respective MCobs (See table 1), there was a significant difference in the negative controls that showed the highest growth rates of the fungi (Table 2).

Table 2. Means of the index of mycelial growth speed (cm day^{-1} ± SD) of phytopathogenic fungi in the minimum concentration observed of clove essential oil and the control treatments.

Phytopatogens	Negative control	Oil on MCobs[1]	Tiram
Fusarium verticillioides	0,85 ± 0,02 a**	0,00 ± 0,00 b	0,02 ± 0,01 c
Macrophomina phaseolina	2,17 ± 0,00 a	0,00 ± 0,00 b	0,00 ± 0,00 b
Macrophomina pseudophaseolina	1,23 ± 0,49 a	0,00 ± 0,00 b	0,00 ± 0,00 b

[1]Minimum concentration determined on in vitro test;
***Letters can be compared in rows; averages with the same letter have no statistically significant difference (Mann-Whitney test, P>0.05).*

According to literature, the phenolic compound Eugenol is the major constituent of clove essential oil, in addition to other chemical compounds such as β-caryophyllene, α-humulene, caryophyllene oxide and eugenyl acetate in lower concentrations [21, 22, 34, 35]. The fungitoxicity of eugenol and other compounds has been reported in some studies [26, 35, 36, 37]. The mechanism of action of clove essential oil is associated with its hydrophobicity, which provides interaction with the wall and lipids of the cell membrane and mitochondria, altering cellular permeability and causing disturbances in its structures.

Also using clove essential oil at concentrations close to or higher than ours, other authors obtained similar inhibition results. For example, under *in vitro* conditions, Costa et al. [23] obtained total inhibition of *Fusarium oxysporum*, *F. solani* and *Rhizoctonia solani* at concentration 0.15%. While in the control of *Sclerotium rolfsii*, Abdel-Kader el al. [38] reached maximum inhibition from the concentration 2.0%. In addition to confirming the antifungal potential of clove essential oil, the results obtained by these studies suggest that the concentration required for inhibition of mycelial growth under *in vitro* conditions will depend on the micro-organism evaluated, justifying the investigation of the minimum concentration in other phytopathogens of economic importance.

Using the essential oil of other plant species on control of *F.verticillioides* and *Macrophomina* spp., other authors obtained significant inhibition results. For example, the total inhibition of *F. verticillioides* was achieved by França et al. [39], Yamamoto-Ribeiro et al. [40] and Bonfin et al. [41] using the Palmarosa (*Cymbopogon martinii*), Ginger (*Zingiber officinale*) and Rosemary (*Rosmarinus officinalis* L.) essential oils at concentrations of 0.2%, 2.500 µg ml^{-1} (0.25%) and 150 µg ml^{-1} (0.015%), respectively. On control of *M. phaseolina*, Khaledi et al. [42] and Ugulino et al. [43] found maximum inhibition using Peppermint (*Mentha piperita* L.)

and Alecrim-da-chapada (*Lippia gracilis*) at concentrations of 2.000 ppm (0.2%) and 0.4 to 1.0%, respectively.

To understand the potential of clove essential oil as a fungicide on *F. verticillioides* and *Macrophomina* spp. we compared its fungitoxic effect with that obtained by a commercial synthetic fungicide. We observed strong inhibition effect of the oil concerning the fungicide at concentrations of 0.05% for *F. verticillioides* and 0.1% for *Macrophomina* spp (Figure 2). This result suggests that under *in vitro* conditions, the fungicide could be replaced by the essential oil.

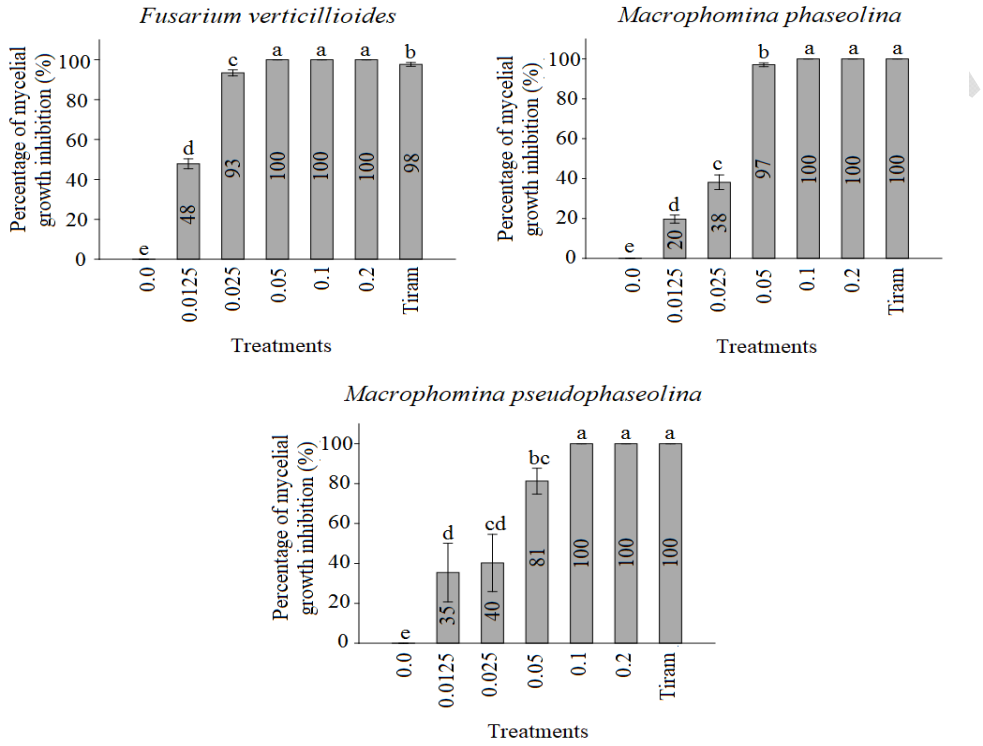

Fig 2. Inhibition of mycelial growth of phytopathogens in the different concentrations of clove essential oil and the control treatments.

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

The microbial control promoted by the essential oils is due to its high chemical complexity, from several constituents [44]. These constituents act through different mechanisms of action in diverse targets simultaneously [38]. These characteristics confer advantage over the use of synthetic fungicide, reducing the possibility of resistance in phytopathogens [45].

3.2 Experiment II: Effect of clove essential oil (*Syzygium aromaticum* L.) on *Fusarium verticillioides* and *Macrophomina* spp. on maize seeds

In the treatment of AG1051 maize seeds, clove essential oil exerted an inhibithory effect from the concentrarion of 0.2% in the fungi *M. phaseolina* and *M. pseudophaseolina*, with a significant reduction in the percentage of infected seeds (Figure 3). On the other hand, essential oil did not promote significant control over *F. verticillioides*.

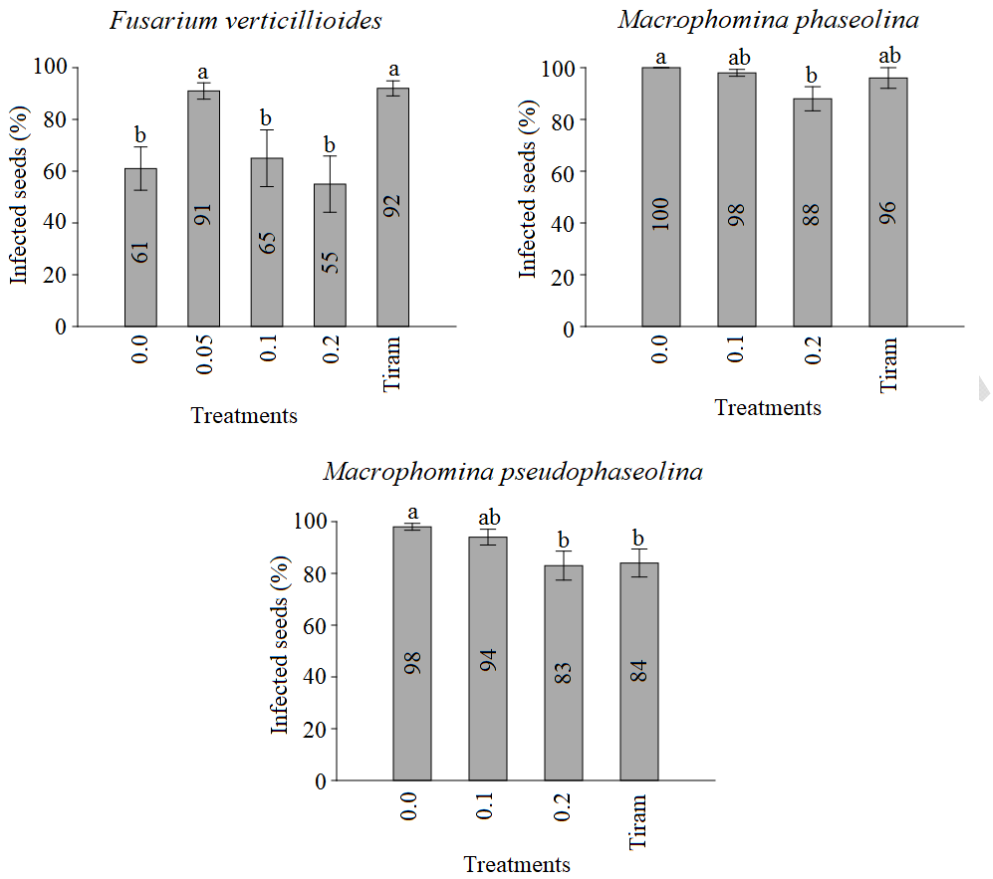

Fig 3. Percentage of infected seed by *Fusarium verticillioides*, *Macrophomina phaseolina* and *Macrophomina pseudophaseolina* after the treatment with the different concentrations of clove essential oil and the control treatments.

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

Using essential oils from other plant species, other authors obtained results similar to ours. For example, evaluating the effect of citronella (*Cymbopogon nardus*) and eucalyptus (*Eucalyptus citriodora*) oils on microflora associated with XGN5320 maize seeds, Brito et al. [46] reached significant inhibition of *Fusarium* sp. at the concentration of 0.15%. Using 'Al Bandeirante' corn seeds treated with the essential oils of Lemon-scented eucalyptus (*Corymbia citriodora*) and eucalyptus (*Eucalyptus camaldulensis*), Domene et al. [47] reported that the oils reduced the incidence of *Fusarium* sp.

The present study showed that concentrations of essential oil used in seed treatment were not enough to significantly reduce the percentage of seeds infected by *F. verticillioides*. Similar results were found by França et al. [39] using the essential oil of Palmarosa (*Cymbopogon martinii*) on *F. verticillioides in vitro* and in the treatment of AG1051 maize seeds. Under *in vitro* conditions, the total inhibition was achieved from the 0.2% concentration, on the other hand, in the treatment of seeds there was significant reduction in the percentage of seeds infected by the fungus from the 3.0% concentration. The authors estimated that the total inhibition could occur when using the essential oil in the concentration of 7.12%.

Fandohan et al. [48] also obtained significant results in the control of *F. verticillioides* in maize seeds. Under *in vitro* conditions, total inhibition of the fungus was obtained from the concentration of 1.3 µL mL^{-1} (0.13%), whereas in seed treatment an increase in concentration was required to reduce the percentage of infected seeds. These results indicate that in the treatment of seeds the increase in the concentration of the oil concerning the *in vitro* experiment becomes required. Possibly we would achieve a greater reduction in the percentage of seeds infected with the increase of the tested concentrations.

The clove essential oil was more efficient reducing seed infection by *F. verticillioides* than the fungicide Thiram above 0.1% of concentration (Figure 3). On the reduction of seeds infected by *M. phaseolina* and *M. pseudophaseolina*, clove oil had a similar effect to that obtained by the fungicide.

Due to the presence of bioactive components with antimicrobial activity, the use of essential oils and plant extracts has become a promising alternative in the control of phytopathogenic fungi [49]. Among the benefits of the use of natural product-based pesticides, it is worth noting their less persistence in the environment [50]. Depending on the plant species used, the essential oil may have low toxicity and, as a result, present the potential to minimize risks to human health and the environment.

In the present study, the growth of *F. verticillioides* and *Macrophomina* spp was paralyzed at concentrations of 0.05 and 0.1%, respectively. In addition, the inhibitions obtained at lower concentrations (0.025 and 0.05%) were quite significant, with a percentage of inhibition higher than 90%. In the treatment of seeds, the oil and essential oil had an effect similar or superior to that obtained by commercial fungicide Tiram.

Ours results could serve as a basis for the formulation of natural defensives based on clove essential oil, which could be implemented in agriculture, minimizing the environmental impacts caused by the exclusive use of chemical pesticides. For this, it is important that safe concentrations of the product are established.

4. CONCLUSIONS

Under *in vitro* conditions, clove essential oil (*Syzygium aromaticum* L.) totally inhibited the mycelial growth of *Fusarium verticillioides* from 0.05% concentration and *Macrophomina phaseolina* and *Macrophomina pseudophaseolina* from 0.1%. In the maize seed treatment, the essential oil at the concentration of 0.2% significantly reduced the incidence of fungi *Macrophomina phaseolina* and *Macrophomina pseudophaseolina*.

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