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# Avaliação do potencial antifúngico do óleo essencial de *Cymbopogon citratus* no controle do fungo *Aspergillus brasiliensis*

# Evaluation of the antifungal potential of *Cymbopogon citratus* essential oil in the control of the fungus *Aspergillus brasiliensis*

# Evaluación del potencial antifúngico del aceite esencial de *Cymbopogon citratus* en el control del hongo *Aspergillus brasiliensis*

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**Filipe da Silva de Oliveira**
ORCID: https://orcid.org/0000-0003-3463-9630
Universidade Federal de Lavras, Brasil
E-mail: filipe.oliveira@estudante.ufla.br
**Carlos Eduardo de Souza Teodoro**
ORCID: https://orcid.org/0000-0001-8591-0502
Universidade Federal Fluminense, Brasil
E-mail: carlosteodoro@id.uff.br
**Pedro Amorim Berbert**
ORCID: https://orcid.org/0000-0003-2480-8162
Universidade Estadual do Norte Fluminense, Brasil
E-mail: pberbert@uenf.br
**Ana Paula Martinazzo**
ORCID: https://orcid.org/0000-0003-0704-2986
Universidade Federal Fluminense, Brasil
E-mail: anapaulamartinazzo@id.uff.br

**Resumo**

O objetivo deste trabalho foi avaliar a atividade antifúngica do óleo essencial de *Cymbopogon citratus* no controle de *Aspergillus brasiliensis* em análises *in vitro* e em grãos de milho armazenados. Foram realizados dois testes *in vitro*, sendo um por difusão em ágar e o outro por microdiluição em poços. As concentrações utilizadas para o ensaio por difusão em ágar foram de 0,2; 0,4; 0,6; 0,8 e 1,0 µL $mL^{-1}$. Para o teste da microdiluição em poços foram

utilizadas as doses de 0,1; 0,2; 0,3; 0,4; 0,5; 0,6; 0,7; 0,8; 0,9; 1,0 e 1,2 µL $mL^{-1}$. Foi avaliado o crescimento micelial ao longo do tempo, tendo sido utilizado o teste de Scott-Knott à 5% de significância para a análise dos resultados. Pelos resultados obtidos, verificou-se que a aplicação do óleo essencial de *Cymbopogon citratus* interfere significativamente no desenvolvimento fúngico da espécie *Aspergillus brasiliensis*. O teste de microdiluição em poços foi utilizado de forma qualitativa, tendo indicado ausência do crescimento em doses a partir de 0,8 µL $mL^{-1}$. Para o ensaio nos grãos de milho, foram utilizadas as dosagens de óleo essencial de 0,5; 0,8 e 1,0 µL $mL^{-1}$, tendo sido armazenados pelo período de 42 dias. Não houve diferença significativa das diferentes doses de óleo essencial ao longo do período de armazenamento, para este ensaio.

**Palavras-chave:** Capim-limão; Grãos armazenados; Fungicida natural.

**Abstract**

The objective of this work was to evaluate the antifungal activity of the essential oil of *Cymbopogon citratus* in the control of *Aspergillus brasiliensis* in analysis *in vitro* and in stored corn kernels. Two in vitro tests were carried out. One of them, by diffusion in agar and the other by microdilution in wells. The concentrations used for the agar diffusion assay were 0.2; 0.4; 0.6; 0.8 and 1.0 µL $mL^{-1}$. The concentrations used for the microdilution test were: 0.1; 0.2; 0.3; 0.4; 0.5; 0.6; 0.7; 0.8; 0.9; 1.0 and 1.2 µL $mL^{-1}$. The mycelial growth was evaluated over time, using the Scott-Knott test at 5% significance. From the results obtained, it was verified that the application of the essential oil of *Cymbopogon citratus* interferes significantly in the fungal development of the species *Aspergillus brasiliensis*. The microdilution test in wells was used in a qualitative way, indicating absence of growth in doses from 0.8 µL $mL^{-1}$. For the corn grain test, the essential oil dosages of 0.5; 0.8 and 1.0 µL $mL^{-1}$ and were stored for 42 days. There was no significant difference in the different doses of essential oil over the storage period for this assay.

**Key words:** Lemongrass; Stored grains; Natural fungicide.

**Resumen**

El objetivo de este trabajo fue evaluar la actividad antifúngica del aceite esencial de *Cymbopogon citratus* en el control de *Aspergillus brasiliensis* en análisis in vitro y en granos de maíz almacenados. Se realizaron dos pruebas in vitro, una por difusión en agar y la otra por microdilución en pozos. Las concentraciones utilizadas para la prueba de difusión en agar fueron 0.2; 0.4; 0.6; 0.8 y 1.0 µL $mL^{-1}$. Para la prueba de microdilución en pozos, se usaron

dosis de 0.1; 0.2; 0.3; 0.4; 0,5; 0.6; 0,7; 0.8; 0.9; 1.0 y 1.2 µL $mL^{-1}$. El crecimiento micelial se evaluó con el tiempo, utilizando la prueba de Scott-Knott con un 5% de importancia para el análisis de resultados. De los resultados obtenidos, se descubrió que la aplicación del aceite esencial de *Cymbopogon citratus* interfiere significativamente con el desarrollo fúngico de la especie *Aspergillus brasiliensis*. La prueba de microdilución en pozos se usó cualitativamente, lo que indica la ausencia de crecimiento en dosis de 0.8 µL $mL^{-1}$. Para la prueba en granos de maíz, se usaron dosis de aceite esencial de 0.5; 0.8 y 1.0 µL $mL^{-1}$, habiendo sido almacenado por un período de 42 días. No hubo diferencias significativas en las diferentes dosis de aceite esencial durante el período de almacenamiento para este ensayo.
**Palabras clave:** Limoncillo; Granos almacenados; Fungicida natural.

## 1. Introduction

According to the growth of population, it is necessary to search for technologies to increase food production. As a result of this search for greater productivity, third generation chemical fungicides are currently being widely used. According to the active ingredient and the amount used in improper ways, damage is caused to consumers and the environment, together with the development of resistance from microorganisms, reducing effective control (Fisher et al., 2015; Silva et al., 2016; Luz et al., 2017).

To this end, alternative ways of inhibiting contamination of food products against fungi, bacteria and insects have been studied, with the use of essential oils (Cherrat et al., 2014; Bomfim et al., 2015; Erland et al., 2015; Farzaneh, et al., 2015; Janatova et al., 2015; Lopez-Romero et al., 2015; Pavela et al., 2015; Prakash et al., 2015; Sharma et al., 2017; Üstüner et al., 2018).

Essential oils are natural, volatile compounds with complex structures. They have a strong odor characteristic and are extracted from aromatic plants, produced by means of their secondary metabolism, and can accumulate in all plant organs. In industries they have different forms of use, from food segments to pharmaceuticals (Ríos, 2000; Knaak; Fiuza, 2010; Machado et al., 2013).

The *Cymbopogon* genus has approximately 180 subspecies, varieties and sub-varieties, with *Cymbopogon citratus* species, together with *C. flexuosus* widely cultivated worldwide (Akhila, 2009; Abdulazeez et al., 2016). Popularly known in Brazil as lemongrass, the species C. citratus, is widely used in phytotherapy as an antispasmodic, anxiolytic and mild sedative (Brasil, 2011; Avoseh et al., 2015; Mosquera et al., 2016; Ekpenyong et al.,

2017). According to the Brazilian Pharmacopeia, the vegetable drug *C. citratus* must present in the composition of its essential oil, at least 60% citral, a mixture of the neral and geranial isomers (Brasil, 2010).

Fungi of the genus *Aspergillus* are known to be the major cause of damage to grain post-harvest in the world. According to Gams et al. (1985), *Aspergillus* section *Nigri*, in which the fungal species *Aspergillus brasiliensis* is classified, has a significant impact on modern society. Although the main source of location for the *Nigri* section is the soil, species in this section have been isolated in several other media, including stored grains (Kozakiewicz, 1989; Abarca et al., 2004; Samson et al., 2004; Boudine et al., 2016; Tournas; Niazi, 2017).

In view of the above, seeking to propose the use of a natural substance for fungal control in the post-harvest of grains, this work aims to analyze the antifungal activity of the essential oil of *C. citratus* for the control of *Aspergillus brasiliensis*, under a specific incubation period in vitro, as well as its activity in the control of microorganism in stored corn grains.

## 2. Material and Methods

The essential oil of *Cymbopogon citratus* was purchased from the cosmetics industry and trade company Argila Indústria & Comércio de Cosméticos.

Analysis of essential oil constituents was performed by gas chromatography - mass spectrometry (GC/MS). The compounds were separated in a fused-silica capillary column with DB-5 stationary phase (30 m long x 0.25 mm internal diameter x 0.25 uM inner film thickness). Helium was used as carrier gas at a flow rate of 1.0 mL $min^{-1}$. The temperature of the injector was hold at 220 °C and the detector at 240 °C. The initial oven temperature was maintained at 60 °C for 2 min and programmed with a heating rate of 3 °C $min^{-1}$ to 240 °C and held for 30 min, in a total analysis time of 91 minutes. The split ratio was 1:20 and the solvent cut-off time was 5 minutes. The sample injection volume was 1 µL, at a concentration of 10,000 ppm, using hexane as solvent.

Compounds were identified by comparing the mass spectra obtained with those of the apparatus database and by the Kovats Retention Index (IK) of each component (Lanças, 1993). The quantitative analysis of the main components of the essential oil, expressed as a percentage, was performed by the peak area integration normalization method, as described by Zhang et al. (2006).

**2.1. Biological Material**

*Aspergillus brasiliensis* strains identified as CCCD AA002 were obtained from the company Didática Scientific Eireli. Cultures were grown in BDA medium (potato, dextrose and agar) in Petri dishes at 30 ºC for seven days. For spore collection, the plates were flooded with 15 mL sterile distilled, and conidia were harvested with a pipette. The spore suspension was adjusted with sterile distilled water to give the final concentration of 4.5 x 106 spores $mL^{-1}$ using a Neubauer chamber. The suspension was stored at 4 ºC until use.

**2.2. *In vitro* test: plate assay**

For the in vitro assay, 20 mL of BDA culture medium were poured into Petri dishes previously sterilized at 121 °C for fifteen minutes in autoclave, containing *C. citratus* essential oil concentrations of diluted in 1% DMSO (dimethyl sulfoxide).

Petri dishes were incubated with 7 mm mycelial discs of both species in the center of the plate. Four replicates were used for each treatment. Two control treatments without essential oil were performed: one with fungus growing on BDA medium only; and the other with fungus growing in BDA medium added with DMSO to evaluate the influence of the surfactant on fungal growth. Because

The Petri plates were incubated in BOD (Biological Oxygen Demand) at 30 °C until the mycelial growth in the control treatments covered the entire Petri dish, with 92 mm diameter, which was considered the end of the incubation time. The colony diameter was record daily with a digital caliper.

The percentage of colony inhibition (PI) was calculated with the following equation (Tatsadjieu et al., 2009):

$$PI = \frac{D_c - D_o}{D_c} \cdot 100$$

$$PI = \frac{D_c - D_o}{D_c} \cdot 100$$

(01)

where:

$D_C$ - diameter of colonies without treatment;

$D_O$ - diameter of colonies treated with essential oil.

### 2.3. *In vitro* test: microdilution

The minimum inhibitory concentration (MIC) of the essential oil (EO) on the fungi studied was determined by serial microdilution in microplate. The doses tested were defined from the results of the *in vitro* test and the following EO doses were tested: 0.2; 0.3; 0.4; 0.5; 0.6; 0.8; 0.9; 1.0; 1.2 μL $mL^{-1}$.

Each dose tested had four replicates in BD medium (potato and dextrose) with the solution containing essential oil, DMSO, and spore suspension ($10^7$), and a control treatment without the essential oil. The plates were kept in a BOD chamber at 35 ºC for 72 h.

After the incubation time, the results were analyzed visually. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of essential oil in which no fungal growth occurred (Pandey, Rai, & Acharya, 2003, Dellavale et al., 2011).

### 2.4. Evaluation of infected corn kernels

For the experiment of growth inhibition of the fungus on corn kernels by the essential oil of *C. citratus*, 1.5 L bottles containing 200 g of kernels were autoclaved at 121 °C for twenty minutes. After cooling, 2.0 ml of *Aspergillus flavus* spore suspension at the concentration of 1.5 x $10^7$ spores $mL^{-1}$ was inoculated to the kernels.

The material was incubated in a BOD at a constant temperature of 30 °C. After 48 hours, the concentrations of 0; 0.5; 0.8, and 1.0 μL $mL^{-1}$ of the essential oil were applied to the kernels stored. The storage times evaluated were 14, 28, and 42 days. At the end of each time, three samples were randomly collected from each treatment and diluted in 0.9% saline solution. A volume of 0.1 mL of the prepared dilution was inoculated into Petri dishes containing approximately 20 mL of Sabouraud agar medium.

The plates were incubated at 28 °C for 72 h in a BOD. Plates containing from 05 to 250 CFU (Colony Forming Unit) were counted and the percent inhibition of growth (PI) was calculated according to Equation 01.

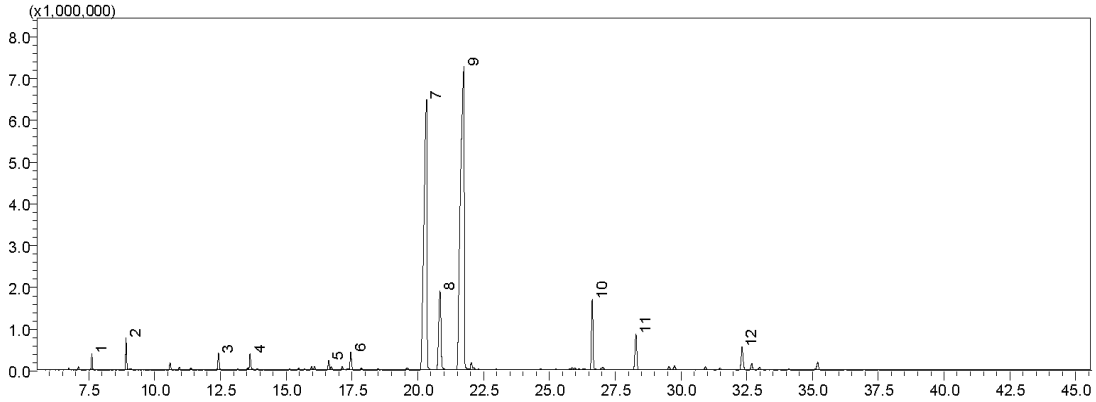
### 2.5. Statistical analysis

The experiment was arranged in a completely randomized design. The results were analyzed by analysis of variance and means compared by the Scott-Knott test at 5% significance level. Data analysis was performed using SISVAR® (Ferreira 2014).

## 3. Results and Discussion

Figure 1 shows the chromatogram obtained in the identification of the components of the essential oil of *Cymbopogon citratus* used. The major component was citral at a concentration of 79%.

**Figure 1.** Chromatogram of the *Cymbopogon citratus* essential oil used in the experiment.




Source: Authors.

It was found that the concentration of citral, a mixture of neral and geranial isomers, in the essential oil of *C. citratus* found in this work of 79%, is in accordance with that presented in the literature. Boukhatem et al. (2014), Ajayi et al. (2016) and Bayala et al. (2018), obtained citral concentration in 73.58, 78.61 and 82.55%, respectively. Furthermore, Negrelle & Gomes (2007) clarify that these values may vary according to the plant's planting location, harvest time, form of extraction, among others.

Table 1 shows the average retention time and Kovats index of the components identified by the chromatogram shown in Figure 1.

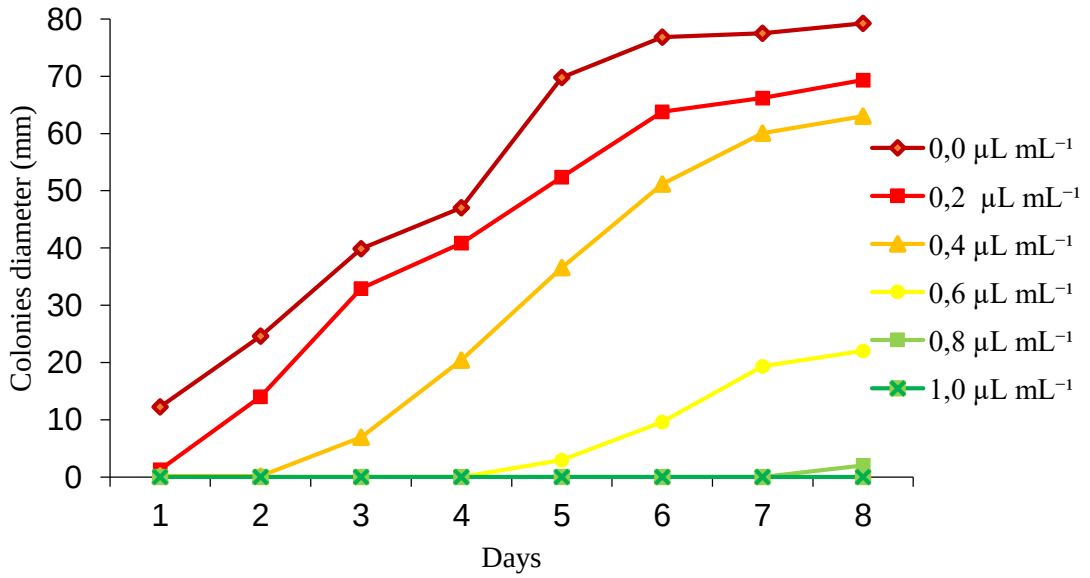
**Table 1.** Main components of *Cymbopogon citratus* essential oil determined by GC- MS.

| Peak | Component | Retention Time* (min) | Kovats Index | | |
|---|---|---|---|---|---|
| | | | Present study | Adams (1995) | Other authors |
| 02 | 6-methyl-5-hepten-2-one | 8,914 | 994 | 992 | |
| 04 | Linalol | 13,630 | 1094 | 1098 | |
| 07 | Neral | 20,338 | 1244 | 1244 | |
| 08 | Geraniol | 20,850 | 1257 | 1257 | |
| 09 | Geranial | 21,754 | 1276 | 1275 | |
| 10 | Geranyl Acetate | 26,634 | 1389 | 1383 | |
| 11 | Caryophyllene | 28,291 | 1429 | - | 1428[(1)] |

*Coluna DB-5. [(1)] Choi (2003).

The inhibitory action, in vitro, of the different doses of essential oil in relation to the control treatment (dose 0) can be seen in Figure 2, which shows the growth of the diameter of the colonies as a function of the incubation period.

**Figure 2.** Effect of different concentrations of *Cymbopogon citratus* essential oil on the mycelial growth of the fungus *Aspergillus brasiliensiss*.

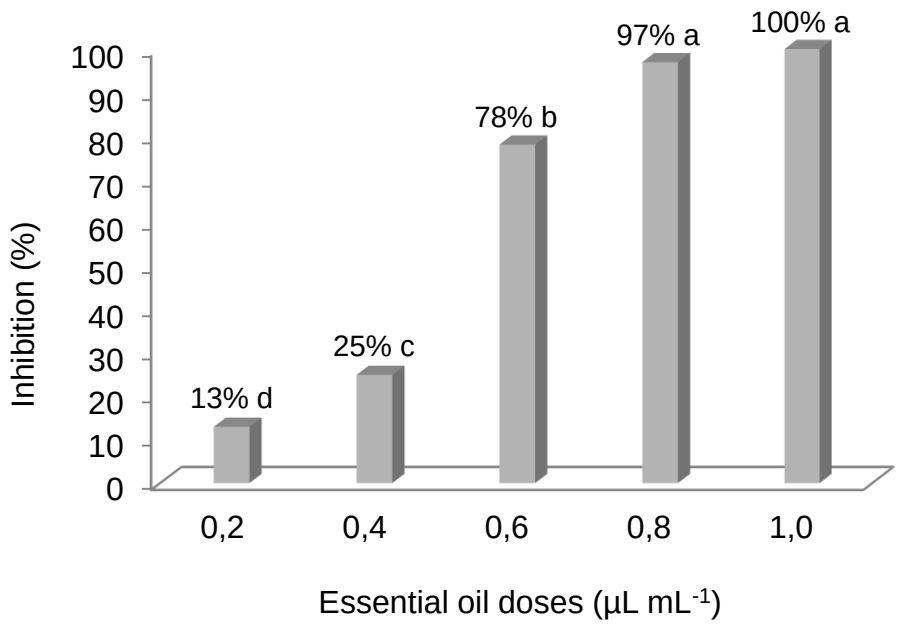



Source: Authors.

Figure 2 shows that *A. brasiliensis* had its development reduced from the dose of 0.6 µL $mL^{-1}$. The dosage of 0.2 µL $mL^{-1}$ was the least efficient, with control over fungal growth only in the first 24 h. The dose of 0.4 µL $mL^{-1}$ showed total inhibition during 48 hours of incubation with reduced control over the period. The 0.6 dose was fully controlled until the fourth day of incubation. At the end of the incubation period, doses of 0.2; 0.4 and 0.6 µL $mL^{-1}$ presented a fungal halo growth diameter of 79, 63 and 22 mm, respectively. At the dose of 0.8 µL $mL^{-1}$, there was a small growth of the fungus (2 mm) only on the eighth day. The dose of 1.0 µL $mL^{-1}$ did not show mycelial growth of the fungus throughout the evaluated period.

Figure 3 shows the percentage of mycelial growth inhibition of the fungus *Aspergillus brasiliensis* at the different doses tested at the end of the incubation period, where the doses of 0.8 and 1.0 µL $mL^{-1}$, remained statistically equal in the control of the microorganism.

**Figure 3.** Percentage inhibition of mycelial growth of *Aspergillus flavus* at different doses (µL $mL^{-1}$) of *Cymbopogon citratus* essential oil after eight days of incubation.




Source: Authors.

Through the disk diffusion test, Ebani *et al.* (2018) found that the MIC of the fungus *Aspergillus fumigatus* by applying the essential oil of *C. citratus* was 0.89 µL $mL^{-1}$. In addition, Rath & Patnaik (2018) and studied the minimum growth inhibitory concentration (MIC) of the fungus *Aspergillus niger* by applying the essential oil of *Cymbopogon citratus*. It was observed by the authors that the minimum inhibitory concentration (MIC) was 0.97 µL

$mL^{-1}$. Boukhatem *et al.* (2014), found that the MIC of the fungus *Aspergillus niger* through the essential oil of *Cymbopogon citratus* was 1.0 µL $mL^{-1}$.

Using the same methodology, Lee (2017) studied the application of the essential oil of *C. flexuosus* in the fungus *Aspergillus flavus*. The minimum inhibitory concentration found by the author was 0.6 µL $mL^{-1}$.

Serial microdilution in microplate was use as a qualitative method, in order to identify the minimum inhibitory concentration (MIC) of the essential oil of *Cymbopogon citratus* on the fungus by applying different doses, stipulated from the results achieved in the in vitro tests. The results obtained are shown in Table 2.

**Table 2.** *Aspergillus brasiliensis* growth * in serial microdilution at different doses (µL/mL) of *Cymbopogon citratus* essential oil.

| **Essential oil doses (µL/mL)** | ***Aspergillus brasiliensis*** |
|---|---|
| 1,2 | - |
| 1,0 | - |
| 0,9 | - |
| 0,8 | - |
| 0,6 | + |
| 0,5 | + |
| 0,4 | + |
| 0,3 | + |
| 0,2 | + |
| 0,1 | + |

* (+) indicates fungal growth and (-) indicates growth inhibited. Source: Authors.

It can be seen from Table 2 that the lowest inhibition dose was 0.8 µL $mL^{-1}$. Considering the minimal differences between doses, the values obtained through the qualitative methodology, present results that are close to those obtained in the plate test, being 1.0 and 0.8 µL $mL^{-1}$.

In addition, Rath & Patnaik (2018) and studied the minimum growth inhibitory concentration (MIC) of the fungus *Aspergillus niger* by applying the essential oil of *Cymbopogon citratus*. It was observed by the authors that the minimum inhibitory

concentration (MIC) was 0.97 µL $mL^{-1}$. Boukhatem *et al.* (2014) found that the MIC of the fungus *A. niger* through the essential oil of *C. citratus* was 1.0 µL $mL^{-1}$.

In addition to in vitro tests, there are authors who test the antifungal activity of essential oils in fungi inoculated on stored grains. Based on the results obtained, the percentage of inhibition of fungal growth inoculated in corn kernels stored for 14, 28 and 42 days was determined by applying essential oil of *Cymbopogon citratus* in the best dosages obtained in vitro (0; 0.8 and 1.0 µL $mL^{-1}$).

In the analysis of variance of the effect of lemongrass essential oil, and of the incubation time on the mycelial growth of the fungus in corn kernels, it was observed that there was no significant effect of the different doses of essential oil (D), as well as for the incubation time (t) and for the interaction (D x t), indicating that the inhibition of the growth of the fungus in corn, with the essential oil of lemongrass, does not depend on the variation of these factors. At doses of 0.8 and 1.0 µL $mL^{-1}$ and in all periods tested (14, 28 and 42 days) there was 100% inhibition in fungal growth, indicating the potential of *C. citratus* essential oil in the control of *A. brasiliensis* in infested corn kernels.

Bozik *et al.* (2017) studied the effect of the essential oil of *C. citratus* on three species of the genus *Aspergillus* spp. isolated oat grains. The authors were able to verify that the dose of 0.5 µL $mL^{-1}$ was sufficient to inhibit the growth of the three fungal species tested.

In addition, using the methodology adapted from the present study, Aoudou *et al.* (2012), studied the effect of the essential oil of *Lippia rugosa* on three species of fungus of the genus *Aspergillus* spp., Inoculated in corn kernels and stored for a period of 30 days. The authors verified that there was a 63% inhibition after 15 days of storage at a temperature of 25ºC, for the dose 0.8 µL $mL^{-1}$. While in the present work, there was 100% inhibition of the fungus *A. brasiliensis* by applying the essential oil of *C. citratus* to corn kernels stored after 14 days.

Also, in order to evaluate the action of fumigated essential oils on grains inoculated with fungi, Kumar *et al.* (2017) studied the control of fumigated essential oils in wheat grains inoculated with the fungus *Aspergillus niger* over three months of storage. The authors obtained 100% inhibition of fungi with the dose of 0.6 µL $mL^{-1}$, while the results of this work show 100% inhibition for the concentration of 0.8 µL $mL^{-1}$of essential oil of *Cymbopogon citratus*, along of six-week storage in corn kernels.

## 4. Conclusions

The essential oil of *Cymbopogon citratus* inhibited 100% of the growth of the fungal species *Aspergillus brasiliensis* in the in vitro control and the minimum concentration dose is 0.8 µL $mL^{-1}$. For the control of the fungus *Aspergillus brasiliensis* inoculated in corn kernels, there was 100% growth inhibition after six weeks with a dose of 0.8 µL $mL^{-1}$.

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**Percentage of contribution of each author in the manuscript**

Filipe da Silva de Oliveira – 35%

Carlos Eduardo de Souza Teodoro – 25%

Pedro Amorim Berbert – 15%

Ana Paula Martinazzo – 25%