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Research Article

IN VITRO EFFICACY OF FIVE ESSENTIAL OILS ON THE MYCELIAL GROWTH OF ASPERGILLUS NIGER ISOLATES FROM ONION BULBS

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ABSTRACT. Onion is a vegetable produced in practically all regions of Burkina Faso. Many efforts are being made to increase national production, but in storage, the loss due to black rot limits the disponibility of local bulbs during the year. The essential oils of *Ocimum gratissimum*, *Ocimum basilicum*, *Cymbopogon citratus*, *Cymbopogon Gigantes*, and *Lippia multiflora*, known to have antifungal properties, were added to a PDA medium and assessed *in vitro* at 500, 1000, 1500 and 2000 ppm concentrations on mycelial growth of isolates of *Aspergillus niger*. After the 4- and 7-day incubation, there was a highly significant difference between the essential oils at concentrations of 500 and 1000 ppm ($p<0.001$), and *Ocimum gratissimum* is considered the best essential oil with an inhibition percentage varying between 47.3 and 89.5%. However, all essential oils showed 100% total inhibition at 1500 ppm and the essential oils of *Ocimum gratissimum* and *Cymbopogon giganteus* demonstrated fungicidal effects at concentrations of 1500 and 2000 ppm, respectively. The intense antifungal activity of *Ocimum gratissimum* oil is related to the presence of thymol, a potent antioxidant responsible for the fungal activity. The activity of *Ocimum gratissimum* oil must also be tested in vivo to develop biopesticide formulations for the control of onion spoilage fungi.

Keywords: *Essential oils, inhibition, fungal isolates, Ocimum gratissimum, fungicide activities.*

INTRODUCTION

Onion are cultivated as a counter-seasonal culture in practically every region of Burkina Faso. It is an important part of the economic and social life in urban, peri-urban, and rural populations [1]. However, most onion producers are confronted by problems of marketing and losses caused by rot and the pre-germination of onion bulbs during conservation. Diagnostic studies conducted on storage onions have demonstrated that fungal diseases are principally caused by the *Aspergillus niger* species [2, 3, 4]. In tropical areas with humid and high climatic conditions, the onion bulbs have very low storage and black rot losses are rapidly increasing to be a major problem for producers [5, 6]. In effect,

black rot due to *Aspergillus niger* has been identified as the most important and damaging fungal diseases of onion bulbs in storage and conservation warehouses [7, 8, 9]. Quadri et al [10] reported that deterioration by the black rot-causing *Aspergillus niger* increased to 80%. In case of severe infection, clusters of black spores form on the surface and between the outermost tunics of onion bulbs. This affects the quality of onion bulbs, often causing their loss from the market. Black rot losses of onion bulbs compromise the storage and conservation periods where in certain production areas, producers have to give at low prices their crops.

In addition to visual alteration, the development of *Aspergillus niger* in the bulbs could produce ochratoxin A (OTA), a mycotoxin responsible for immunosuppressive, carcinogenic, and teratogenic effects [11, 12]. Presently, the existence of *Aspergillus niger* on conserved onion bulbs is a serious problem regarding sanitary safety, nutritional quality, and economic loss for producers. Few studies are known on methods to prevent a protection onion bulb rot in conservation. Essential oils of *Ocimum gratissimum, Ocimum basilicum, Cymbopogon giganteus, Cymbopogon citratus* and *Lippia multiflora* showing antifungal properties would be an alternative for the prevention and protection of onion bulbs in conservation [13, 14, 15]. This study was conducted to assess the efficacy of five essential oils *in vitro* on the mycelial growth of *Aspergillus niger* isolates identified from spoilage bulb samples in a conservation warehouse in Burkina Faso.

MATERIALS AND METHODS

Vegetable material

The vegetable material is constituted of five (05) aromatic plants namely *Lippia multiflora, Ocimum bacilicum, Ocimum gratissimum, Cymbopogon citratus,* and *Cymbopogon giganteus*. These were collected in the peripheral areas of Bobo Dioulasso and *Lippia multiflora* was collected in Loumbila, a peripheral area of the city of Ouagadougou/Burkina Faso.

Test organisms

The test organisms are constituted of four isolates of *Aspergillus niger* from the mycotheque of the Clinical Laboratory of Plants of the NAZI BONI University of Bobo Dioulasso. These isolates were extracted from samples of alteration bulbs from Sourou, Kongoussi, Yako and Korsimoro, the principal production areas of Burkina Faso (Table 1).

Table 1. *Distribution of Aspergillus niger isolates by locality of a sample of altered bulbs*

Fungal species	Locality	Fungal Host	Code
Aspergillus niger	Sourou	violet de galmi	*A. niger*_vgl_sourou
	Kongoussi	safari	*A. niger*_saf_kongoussi
	Yako	safari	*A. niger*_vgl_yako
	Korsimorro	violet de galmi	*A. niger*_vgl_korsimoro

Extraction of essential oils

Aromatic plants have been extracted by hydrodistillation in 50 liters capacity alambic. Each extraction process has a duration of 2 to 3 hours after the first drop of water is mixed with essential oil. The decantated was use to recuperate essential oils extracted and the mixture was kept at the laboratory ambient temperature until they are used in anti-fungal tests [16].

Cultivation of fungi

It was conducted by subculturing Petri dishes containing potato dextrose agar (PDA) medium [17, 18, 19]. Mycelial explants were placed into the centre of Petri dishes containing PDA medium and the dishes were then closed with paper scotch tape. After inoculation, the inoculated Petri dishes are incubated under 12 h of ultraviolet light alternated with 12 h of the dark at 25 °C for 7 days. The seven-day-old isolates were used for biological tests.

In vitro antifungal activities of essential oils on the mycelial growth of fungal strains by the poisoning method

The *in vitro* anti-fungal activity was tested using the mixture of poisoning PDA medium with essential oil. The method is based on the determination of the minimal inhibitory concentration (MIC) on previously prepared PDA-essential oil (HE) media, defined as the lowest concentration that inhibits all growth visible to the visual perception after an incubation period [20, 21].

Preparation of PDA-essential oil media

PDA media was prepared in an autoclaforing 20 min at 121 °C. After the temperature of these PDA media was decreased in a bain-marie to the superfusion temperature, the essential oils were added to final concentrations of 500, 1000, 1500, and 2000 ppm. The PDA-essential oil mixture is emulsified under magnetic agitation for 20-30 min, then transferred into Petri dishes based on others by treatment. The dishes with PDA modified with essential oil were kept under the hood at ambient temperature to permit better solidification. The control without essential oil was conducted under the same conditions.

Inoculation and incubation

To test *in vitro* antifungal activity, mycelial explants were obtained on the mycelial growth front of 7-day-old colonies using a 5 mm diameter punch. Each mycelial explant is applied in the middle of the Petri dish that contains a solid PDA-essential oil medium, by using a curved needle. For each test and each concentration, we used four dishes representing the four repetitions. The inoculated Petri dishes (assay and control) were closed with paper scotch tape and incubated under 12h of near-ultraviolet light alternated with 12 h of the dark at 25 °C for 7 days.

Evaluation of inhibition potential of essential oils

Mycelial growth was evaluated by measuring the two perpendicular diameters (verso of the box) of mycelial growth at 4 and 7 days after incubation (DAI). The increase (average of all values) was deducted from the explant diameter (0.5 cm) from the

measured values. The data obtained were used to determine the Percentage Inhibition (PI) based on the following formula [22]:

$$PI\ (\%)=[(D - Di)/D]x100$$

D is the diameter of mycelial growth in a medium without essential oil (control) and Di is the diameter of mycelial growth with essential oil.

Evaluation of fungistatic (CFS) and fungicide (CMI) concentrations of essential oils analyzed

After evaluation of inhibition activities of the essential oils at 7 days after incubation (DAI) on the growth of mycelial explants, the dishes with no growth were chosen to determine the fungistatic or fungicidal effects of the essential oils tested. To achieve this result, the mycelial explants were transferred from dishes, and the inhibition was total to new dishes containing a new PDA culture medium without essential oil. These new inoculated dishes were then placed in the incubator at a temperature of 25 °C under an alternating cycle of near-ultraviolet light and dark (12h /12h) during 7 DAI, if mycelial growth was observed, treatment with essential oil is declared fungistatic (CFS) anon in the anon, in any case, it is declared fungicide (CMF) [23].

Statistical analyses of data

The data were analyzed with R statistic software (www.r-project.org) version 4.0.3. In the global analyses, we have used the multivariate analysis of variance. The values of $p \leq 0.05$ are considered significant. The experiments were repeated four times (n=4). The statistical tests based on univariate analysis of variance were completed using multiple comparisons of means by Duncan's tests at 5% for the determination of signification levels and multiple comparisons. The statistical tests were conducted with the packages (ADE4, stats, stats4, agricolae, tidyverse, ggpubr, rstatix, funModeling, drc, datarium, mvnormtest, emmeans, multcomp, gplots, Rmisc).

RESULTS AND DISCUSSION

In vitro antifungal activities of essential oils on mycelial growth of Aspergillus niger isolates

Analyses of variance

Statistical data from the analysis of variance univariate at a 5% threshold, demonstrated that the inhibition of mycelial growth is significantly different between incubation dates with a probability $p=0.007<0.05$. But there are no significant differences between the sensibility of *Aspergillus niger* isolates for any incubation data used to evaluate the anti-fungal activities ($p>0.05$). In contrast, there were highly significant differences in inhibition between treatments with essential oils and concentrations used ($p<0.001$) (Tables 2 and 3).

Table 2. *Analyze of variance of inhibition percentage at 4 DAI in the function of tested variables*

Variable	Sum of squares	degree of freedom	Mean square	F-value	Probability
Isolates	4 446	3	14 82,1	1,517	0,211
Residual	2 305 337	236	976,8		
Treatment	26 365	4	6 591	7,425	$1{,}2.10^{-5}$***
Residual	208 618	235	1 109		
Concentration	157 685	1	157 685	485,5	$< 2.10^{-16}$***
Residual	77 298	238	325		

***: highly significant difference.

Table 3. *Analyze of variance of inhibition percentage at 7 DAI in the function of tested variables*

Variable	Sum of squares	degree of freedom	Mean square	F-value	Probability
Isolates	3627	3	1209	0,848	0,465
residual	336297	236	1425		
Treatment	32071	4	8018	6,12	$1{,}06.10^{-4}$ ***
residual	307853	235	1310		
Concentration	239311	1	239311	566,1	2.10^{-16} ***
Residual	100613	238	423		

***: highly significant difference.

Comparative analysis of analysis fungal activities of in vitro essential oils on mycelial growth of Aspergillus niger isolates

Statistical data for univariate analyse of variance are presented in fig. 1 at α=5% demonstrates that there exists a highly significant difference between anti-fungal activities of essential oils tested at 500 and 1000 ppm at p-values of $1{,}69.10^{-10}$ (F = 22,11; ddl=35) and $P=5{,}82.10^{-15}$ (F=58,02; ddl=35) respectively. In effect, statistical data obtained from Duncan's test showed that independently of incubation date at 500 and 1000 ppm concentrations, the essential oil of *Ocimum gratissimum* demonstrated better anti-fungal activity with percentages of inhibition varying, respectively, from 47.3% to 52.5% and 88.3% to 89.5%. At a concentration of 500 ppm, it was followed by *Cymbopogon citratus* and *Ocimum basilicum* essential oils, whose inhibitory effects were moderately active at 4 DAI with inhibition percentages, respectively, of 35.5% and 27.1%, and at 7 DAI, not actives with inhibition percentages of 19.4% and 2.4%. However, the essential oils of *Cymbopogon giganteus* and *Lippia multiflora* were not actives and the percentage of inhibition varied between 5.5% and 15.9% at any incubation date. At 1000 ppm, the essential oil of *Lippia multiflora* showed very active and dominant inhibitory effects, similar to the essential oil of *Ocimum gratissimum* at both 4 and 7 days with inhibition percentages varying between 89.4% and 91.5%. Whereas *Cymbopogon giganteus* essential oil was very active and statistically dominant at 4 DAI with an

inhibition percentage of 76.3% and moderately active at 7 DAI with an inhibition percentage of 47.2%. However, essential oils of *Cymbopogon citratus* and *Ocimum basilicum* were moderately active at 4 DAI, with 43.3% and 34.6% inhibition percentages respectively, and not active at 7 DAI with 21.1% and 17.7% inhibition percentages respectively. By the opposite, there was not one significant difference in anti-fungal activities of essential oils at 1500 ppm because all essential oils showed 100% inhibitions from the concentration of 1500 ppm.

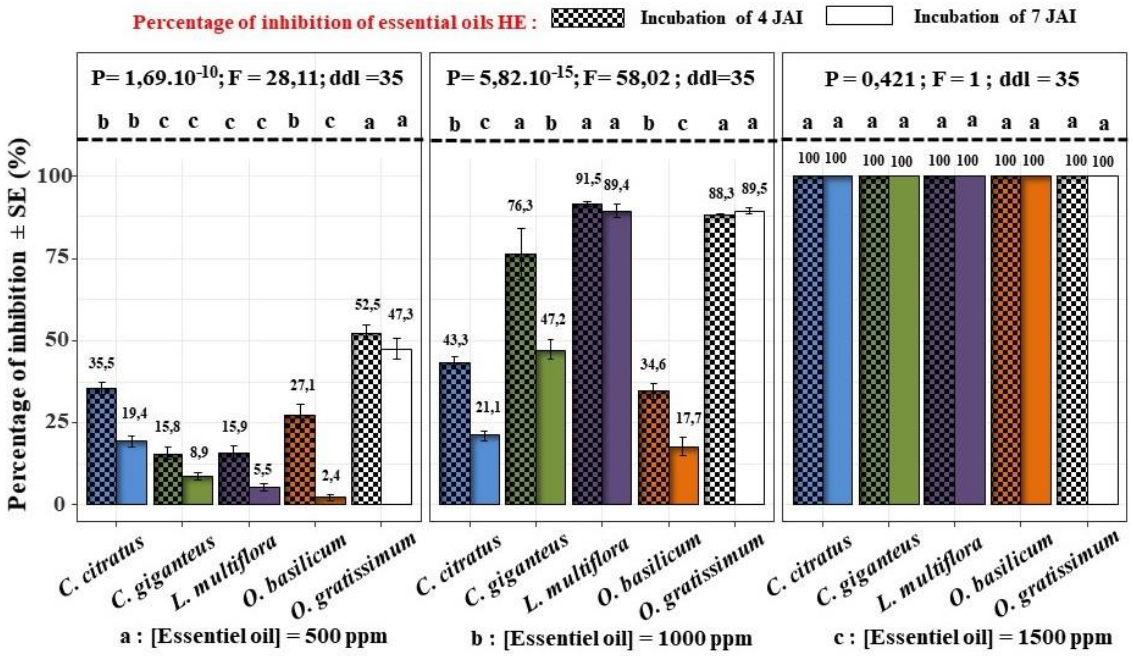

Fig. 1. *Anti-fungal activities of essential oils on mycelial growth at 4 and 7 days from the identified isolate of Aspergillus niger of Sourou spoilage bulbs.*

Antifungal screening of essential oils on mycelial growth of Aspergillus niger isolates of sourou

Statistical data from univariate ANOVA on the inhibition of mycelial growth of Sourou isolate are presented in fig. 2 showed that α=5%, and independently of essential oil and incubation date, there is a highly significant difference between concentrations of essential oils tested at $p<0.001$. In *vitro* anti-fungal screening of essential oils and independently of applied incubation date, the 1500 ppm concentration showed 100% total inhibition compared with concentrations of 500 and 1000 ppm showing variable anti-fungal potential in the function of essential oils and incubation date. During the *in vitro* anti-fungal screening of essential oils and independently of the applied incubation date, the 1500 ppm concentration showed a total inhibition at 100% compared to concentrations of 500 and 1000 ppm that showed a variable anti-fungal potential dependent on the essential oil and the incubation date. However, at a concentration of 500 ppm, the essential oil of *Ocimum gratissimum* was active with inhibition percentages comprised between 47.2% and 52.5% and that of *Lippia multiflora* was not active with

an inhibition percentage comprised between 5.5% and 15.9%. The anti-fungal potential of *Cymbopogon giganteus* essential oil tested with 1000 ppm was very active at 4 DAI with inhibition of 76.3% and moderately active at 7 DAI with inhibition of 47.3%. At the concentration of 500 ppm, the anti-fungal potential of *Cymbopogon giganteus* essential oil was not active with inhibitions that varied between 8.9 % and 15.8 %. Additionally, in the anti-fungal screening of *Cymbopogon citratus* and *Ocimum basilicum* essential oils, anti-fungal activities at 500 and 1000 ppm was moderately active at 4 DAI with percentages of inhibition comprised between 27.1% and 43.3% and not active at 7 DAI with percentages of inhibition comprised between 2.4% and 21.1%.

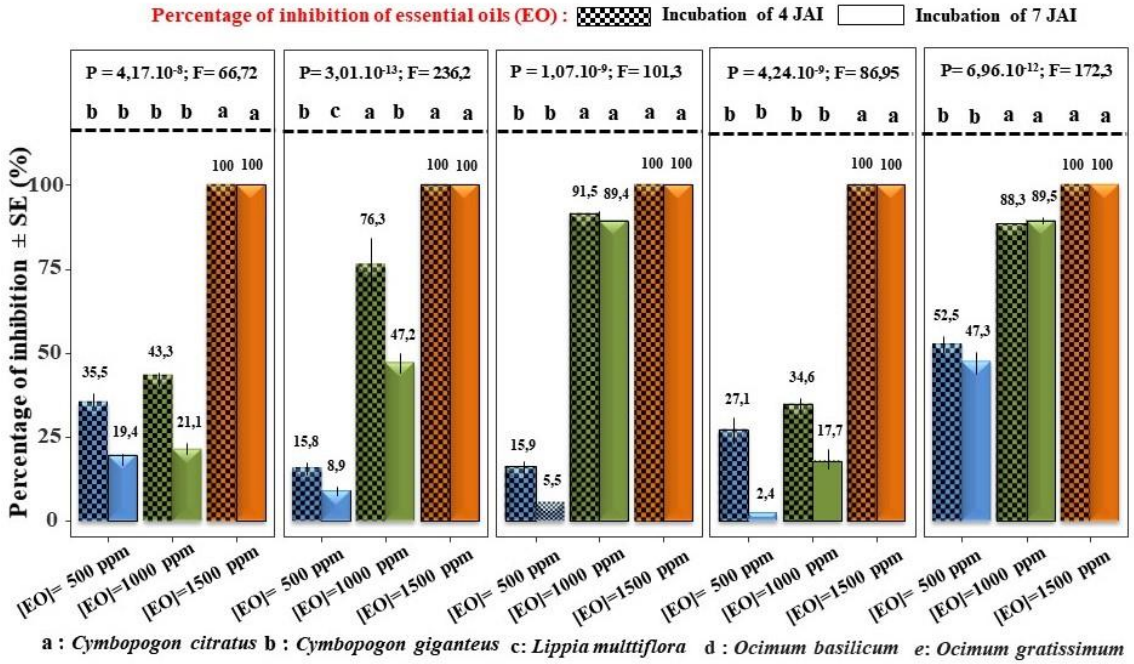

Fig. 2. *Screening of anti-fungal activities of essential oils on mycelial growth at 4 and 7 JAI of Aspergillus niger isolate identified from spoilage bulbs of Sourou*

Fungistatic (CFS) and fungicidal (CMI) activities of essential oils on the mycelial growth of Aspergillus niger isolate of sourou

Results presented in Table IV on the fungicide and fungistatic activities of the essential oils assayed at 1500 and 2000 ppm indicated that essential oils of *Ocimum gratissimum* and *Cymbopogon giganteus* produced fungicide effects on the mycelial growth of *Aspergillus niger* isolates at minimum fungicides concentrations of 1500 and 2000 ppm respectively. In effect, mycelial explants after their inhibition on PDA medium modified by essential oils of *Ocimum gratissimum* and *Cymbopogon giganteus* at concentrations of 1500 ppm and 2000 ppm respectively and their transferred to new PDA medium without essential oils did not show regeneration of mycelial growth of the *Aspergillus niger* isolates tested. The essential oils of *Ocimum basilicum*, *Cymbopogon citratus*, and *Lippia multiflora*, however, presented fungistatic activities because there was a regeneration of the mycelial growth of their explants transfer to a new PDA medium without essential oil.

Table 4. *Fungistatic (CFS) and fungicide (CMF) activities in ppm of essential oil of five aromatic plants*

Treatments / fungicide activity		**Isolates of *Aspergillus niger* tested**			
		A. niger vgl_sourou	***A. niger*** saf_kgssi	***A. niger*** vgl_korshi	***A. niger*** vgl_yako
Ocimum gratissimum	CMI (ppm)	1500	1500	1500	1500
	CMF (ppm)	1500	1500	1500	1500
Fungicide activity		**Fongicide**	**Fongicide**	**Fongicide**	**Fongicide**
Ocimum basilicum	CMI (ppm)	1500	1500	1500	1500
	CMF (ppm)	>2000	>2000	>2000	>2000
Fungicide activity		**ND**	**ND**	**ND**	**ND**
Cymbopogon giganteus	CMI (ppm)	1500	1500	1500	1500
	CMF (ppm)	2000	2000	2000	2000
Fungicide activity		**Fongicide**	**Fongicide**	**Fongicide**	**Fongicide**
Cymbopogon citratus	CMI (ppm)	1500	1500	1500	1500
	CMF (ppm)	>2000	>2000	>2000	>2000
Fungicide activity		**ND**	**ND**	**ND**	**ND**
Lippia multiflora	CMI (ppm)	1500	1500	1500	1500
	CMF (ppm)	>2000	>2000	>2000	>2000
Fungicide activity		**ND**	**ND**	**ND**	**ND**

ND: not determined

In vitro anti-fungal screening of five essential oils was tested by using the method of poisoning PDA medium with by five essential oils at 500, 1000, 15,00, and 2000, ppm concentrations on the mycelial growth of *Aspergillus niger* isolates. In effect, the inhibition percentage of essential oils tested at 500 ppm and 1000 ppm concentrations on mycelial growth developed progressively and in heterogeneity between *Aspergillus niger* isolates depending on treatment and incubation period. However, the essential oil of *Ocimum gratissimum* was the best essential oil because of the important inhibition effects at 500 and 1000 ppm concentrations. Contrary to this, independent of the incubation date, essential oils tested at 1500 and 2000 ppm concentrations presented 100% total inhibition of mycelial growth of *Aspergillus niger* isolates. Subcultures conducted following 100% inhibition at 1500 and 2000 ppm concentrations permitted to observe the fungicide effect of essential oils of *Ocimum gratissimum* and *Cymbopogon giganteus* with minimum fungicide concentrations (CMF) respectively of 1500 ppm and 2000 ppm on all isolates of *Aspergillus niger* tested. For minimum inhibitory concentrations of 1500 and 2000 ppm of essential oils of *Ocimum basilicum, Cymbopogon citratus,* and *Lippia multiflora*, the minimum fungicide concentrations could not be determined for any isolates of *Aspergillus niger* studied. The sensitivity of isolates of *Aspergillus niger* vis-a-vis the inhibitory action of essential oils could be related to bioactive molecules that were present in the culture media at the moment of anti-fungal tests and that characterize each essential oil. The fungicide effect of essential oils tested is justified by an absence of mycelial growth after the subculture of the inhibited explant on the PDA medium without essential oils. The examination of the inhibitory effects and the fungicidal properties of essential oils revealed that the inhibitory/fungicide effect could be related to different concentrations used because high concentrations of essential oil would induce membrane rupture of microorganisms [24]. Therefore, the intense anti-fungal activity of *Ocimum gratissimum* essential oil could be associated with its high content of thymol, a potent

antioxidant that has known to have anti-fungal activities [25, 26, 27]. Indeed, several studies have demonstrated the antifungal efficacy of terpene phenols and particularly thymol and/or carvacrol, which possess a very large specter of anti-fungal activities [28, 29, 30]. In effect, phenolic terpenes are active against fungi through different mechanisms based, firstly, on the inactivation of the fungal enzymes containing the SH group in their active site and, secondly, by a fixation on amine and hydroxylamine groups of microbial membrane proteins, resulting in the alteration of permeability and leakage of intracellular constituents [31, 32, 33]. However, research by Trombetta et al [34] suggested that thymol is responsible for the inactivation of enzymes, including those involved in energy production and synthesis of structural components. Additionally, some factors could limit the fungicide potential of other essential oils tested for inhibition of *Aspergillus niger* isolates. This concerns their volatile character, which would dissipate between the time of manipulation and the period of incubation, and would reduce the efficacy of certain essential oils during evaluation. It is also important to note that some bioactive compounds of essential oils are heat unstable and thus are rapidly degradable on dilution with PDA culture medium at superfusion temperature (45 °C to 50 °C).

CONCLUSION

The anti-fungal activity was evaluated concerning the efficacy of essential oils of *Lippia multiflora, Cymbopogon gigantes, Ocimum gratissimuum, Ocimum basilicum,* and *Cymbopogon citratus* on the mycelial growth of *Aspergillus niger* isolates. The effects of five essential oils were tested with success on the mycelial growth of *Aspergillus niger* isolates at 4 and 7 DAI to achieve inhibition of 100% at 1500 ppm concentration. In the total inhibition of *Aspergillus niger* isolates, the essential oil of *Ocimum gratissimum* presented the better inhibitory/fungicidal activity with a minimum fungicide concentration (CMF) of 1500 ppm, followed by the essential oil of *Cymbopogon giganteus* that showed the better fungicide properties at CMF of 2000 ppm. The essential oils of *Ocimum basilicum, Lippia multiflora,* and *Cymbopogon citratus*, showed only fungistatic properties because the CMF was not determined.

Conflict of Interest. The author declared that there is no conflict of interest.

Authorship Contributions. Concept: A.R.O., S.B., L.P.O., I.S., R.H.C.N., I.S., Design: A.R.O., S.B., Data Collection or Processing: L.P.O. I.S., Analysis or Interpretation: L.P.O., I.S., Literature Search: A.R.O., S.B., Writing: O.A.O., R.H.C.N., I.S.

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