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Impact of lemongrass essential oil on growth, mycelial biomass, enzymes activities, and aflatoxins production of *Aspergillus* fungi from smoke fish in Cameroon urban markets.

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Abstract
Lemongrass Essential oil (Eos) is a plant extract with known antifungal properties potentially important for the bio-control of dry products in store. This study focuses on the capacity of lemongrass Eos to reduce the growth and mycelial biomass production, amylase and cellulase activities, and control of the aflatoxins production of Aspergillus species. The food poisoning technique was used to assess the growth inhibition and biomass production capacity of fungi in potato dextrose agar and potato dextrose broth medium. The inhibition potential of Eos on amylase and cellulase activity was evaluated using starch and carboxymethyl cellulose respectively as substrates. The bio-control capacity of Eos on aflatoxins production of Aspergillus species was tested on potato dextrose agar and coconut milk agar. Results show that neither mycelial growth nor biomass production was recorded with the Aspergillus species tested at the concentration of 0.4 µ/ml. The minimum inhibitory concentration was 0.1 and 0.4µl/ml respectively for Eo2 and Eo1. The inhibition rate of lemongrass Eos is a function of the type as Eo2 from mycorrhizal plants at 0.1 µ/ml is totally active on the tested species except for A. tamari compared to Eo1 from non-mycorrhizal plants, mostly active at 0.2 µ/ml. No amylase activity was recorded for the species of Aspergillus tested however cellulase activity was recorded with all the species involved. Intense activity was noted after supplementing the media with Eos, with the best attributed to Eo2 followed by Eo1. Of the three species of Aspergillus tested, two were aflatoxins producers. Supplementation of the media with both lemongrass Eos totally inhibits the production of aflatoxins by the involved microorganism. Lemongrass Eos is indicated as biocontrol of Aspergillus fungi, and those from the mycorrhizal plant are better. Cellulase and aflatoxins are tools to test the bio-properties of plant extract including Eos.
Keywords*: AM fungi; enzymes; lemongrass essential oil; mycelial growth; smoke fish. .*

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I. Introduction

Fisheries sectors and resources are credited with the important feeding capacity of populations worldwide, the significant contribution to promoting socioeconomic growth, and the improvement of livelihoods of marginalized (Béné et al., 2016; Chan et al., 2019). A growing belief progressively supports that fish is the principal source of animal protein for more than 30% of Africa's human population representing more than 200 million people (Tran et al., 2019; Muringai et al., 2022). Fresh fish after capture is a perishable commodity in the tropics in the absence of adapted conservation equipment (Degnon et al., 2013). Various approaches in line with the reduction of post-harvest losses have been developed many years ago, including salting, drying, and smoking. In the coastal areas of Cameroon, a large part of fish caught is always smoked or dried to improve shelf life before

carrying to the consumers' area (Folorunso *et al*., 2006). Despite the multitude of those methods with accepted results, smoked dry fish remains a perishable commodity during storage due to the post-smoking management practice, the development of pests and fungal contaminants, and the storage environment (Molaei *et al.,* 2020; Taherzadeh-Shalmaei *et al.,* 2021; Parker and Pontin 2021). It has been reported that smoke-dried fish is often contaminated with microorganisms including bacteria, yeasts, and molds while passed through the processing units up to markets (Ngo Oum et al., 2021; Hungerford et al., 1998). Several fungal agents associated with the contamination of smoked and smoke-dried fishes, such as *Aspergillus,* are known to be opportunistic pathogens, causing varying degrees of health problems to both animals and humans. In between, some pathogenic agents colonizing different types of fish are also toxigenic (Hungerford et al., 1998; Swaminathan et al., 1998). Synthetic chemicals are the best alternative used by processors and traders to shorter losses with positive results. However, crazy consequences on consumers' health were recorded including disturbance of hormone balances, development of cancers and allergies, as well as the degradation of the environment (Rochester *et al*., 2015). Arising concerns about the side effects of synthetic pesticides on human health gradually motivates consumers to pay a premium price for natural and organic fresh products (Rahman et al., 2021). Around 500,000 species account for plant populations, with a relatively small percentage (1 to 10%) of these used as foods by both humans and other animal species (Moerman, 1996). Some of those plants are essential oils (EOs) producers which are gaining interest as food additives, widely accepted by consumers because of their relatively high volatility, ephemeral and biodegradable nature. More than 17,000 aromatic plant species commonly belong to angiosperms families Lamiaceae, Rutaceae, Myrtaceae, Zingiberaceae, and Asteraceae (Regnault-Roger et al., 2012). About 60% of essential oil derivatives studied up to now are credited with properties of fungi inhibition (Wu et al., 2023). *Cymbopogon citratus* also called Lemongrass is one of approximately 55 species of grasses belonging to the *Poaceae* family. This family of plants is generally recognized as an essential oil producer. *Cymbopogon citratus* Eos is the store of various chemical compounds including Néral, Myrcène, Géranial, and Géraniol, some sesquiterpenes and more with various concentrations according to the plant's environmental growing (Fokom *et al*., 2019; Muturi et al., 2020; Mukarram et al., 2021; El-Kased and Kersh 2022).

This study was addressed to study the influence of two Lemongrass Eos on the growth inhibition and mycelial biomass production, the inhibition of amylase and cellulase activities, and the control of the aflatoxins production of *Aspergillus* species from smoke dry fish in the Cameroon urban market.

II. Materials and methods

2.1 Fungal and Essential Oils materia.

The *Aspergillus* fungi used in this study were obtained from the collection of microorganisms at the Soil Microbiological Laboratory, the Biotechnological Center of Nkolbisson, the Yaoundé I University, Cameroon. Details related to those fungi are in Ngo oum et al., (2021). They include *A. fumigatus, A. flavus, A. niger, A. nomius, A. oryzae, A. ochraceus,* and *A. tamari*. Two essential oils (EOs) were obtained from the same Laboratory, and includ 1 from the none mycorrhizal lemongrass plant and 2 from the mycorrhizal lemongrass plant. Details regarding Eos are provided in Fokom et al., (2019).

2.2 Determination of the antifungal activity of Eos.

Evaluation of the antifungal activity of Eos was carried out using the food poisonous technique as described by Adjou *et al*., (2012). Specific initial concentrations (0.1, 0.2, and 0.4 μl/ml) were prepared by adding an appropriate amount of Eos and 0.5% (v/v) Tween 80 to cool molten PDA (45◦ C) followed by manual rotation in a sterile Erlenmeyer flask to disperse the oil in the medium. Twenty milliliters of medium were distributed in sterile Petri dishes (9 cm in diameter) taking care to avoid trapping air bubbles. The medium was left to consolidate at room temperature for approximately one hour. Fungi were first activated with a serial replication in potato dextrose agar (PDA) media at 28°C for 7 days before being used for the study.

Agar discs with mycelium (6 mm in diameter) were cut out around the actively growing regions of purebred 7-day cultures using a sterile cork borer and aseptically inoculated in the center of Petri dishes. Control plates (without Eos) were inoculated following the same procedure. Three replicates were maintained for each treatment and the plates were incubated at 28°C for 5 days before the diameter of the fungal colony was taken in each plate. The inhibition percentage of the mycelial growth of the fungi was calculated using the procedure of Philippe *et al.,* (2012).

Inhibition of mycelial growth (%) = $(dc - dt/dc) \times 100$,

Where dc is the mean diameter of the colony in the control plate, dt is the mean diameter of the colony in the test plate.

2.3. Determination of Minimum Inhibitory and Fungicidal Concentration.

Minimum inhibitory concentration (MIC) was defined as the lowest concentration of Eos at which no growth occurred. To establish whether Eos had a biocidal effect on the test fungi, the minimum fungicidal concentration (MFC) of Eos on the test fungi was assessed as follows. The inhibited fungal discs of the Eos treated plates were reinoculated into freshly prepared PDA petri plates without Eos and their growth revival was observed after incubation for 72 hours at 28°C. MFC was taken as the lowest concentration of the Eos at which no growth occurred on the plates after subculturing (Adjou *et al*., 2012).

2.4. Effect of Eos on dry mycelium weight

The effect of the essential oil on the dry mycelium weight of the fungi was measured by the method of Dikbas et al., (2008). The Eos was dissolved and diluted tenfold in 5 mL L−1 Tween 20 and transferred into Erlenmeyer flasks with 50 mL of PDB medium to obtain oil concentrations of 0 (control), 0.1, 0.2, and 0.4 µL mL−1. A fragment of active growing mycelium of fungi was inoculated into each flask and the flasks were incubated on a rotary shaker at 0.58 × g at 25 ∘C. The mycelia were harvested on day 5 by centrifugation at 3260 × g for 15 min and precipitates were washed twice by dipping them in distilled sterile water and filtering for 30 min. The dry mycelia were weighed after drying the wet mycelia at 60 ∘C for 12 h. Each treatment consisted of three replicates and the experiments were performed three times.

2.5 Effect of Eos on Amylase Production

The fungal strain was grown on Czapek-Dox agar media, supplemented with 1% of starch and an appropriate volume of Eo to make 0,2 µl/ml. Control Petri plate with 1% starch and no Eo was simultaneously made to follow up the experiment. After 7 days of incubation, the plate was flooded with an aqueous iodine solution (1 % w/v) and incubated for 15 min. The iodine solution was poured off, and the plate was further treated by flooding it with NaCl solution (1 M) for another 15 min. The formation of a clear zone of hydrolysis around colonies indicated starch degradation. The diameter of the clear zone around the colony was measured in order to classify the Eo according to their capacity to affect amylase activity. The largest diameter was assumed to contain the highest activity (Varalakshmi *et al*., (2009).

2.6 Effect of Eos on Cellulase production

The fungal strain was grown on Czapek-Dox agar media, supplemented with 1,5% Carboxymethyl Cellulose (CMC) and an appropriate volume of Eo to make 0,2 µl/ml. Control Petri plate with 1,5% CMC and no Eo was simultaneously made to follow up the experiment. After 7 days of incubation, the plate was flooded with an aqueous solution of Congo red (1 % w/v) and incubated for 15 min. The Congo red solution was poured off, and the plate was further treated by flooding with NaCl solution (1 M) for another 15 min. The formation of a clear zone of hydrolysis around colonies indicated cellulose degradation. The diameter of the clear zone around the colony was measured in order to classify the Eo according to their capacity to affect cellulase activity. The largest diameter was assumed to contain the highest activity (Ariffin et al., 2006).

2.7 Effect of Eos on aflatoxin production.

Potato dextrose agar (PDA) and Coconut agar Media (CMA) were used for the detection of aflatoxin based on fluorescence Davis et al., (1987). After sterilization, an appropriate amount of Eo was added to the media to make 0.2µl/ml concentrations before introduction in Petri plates. Control plates without EO were mated to follow the experiment. A fragment of actively growing mycelium of fungi was inoculated and the plates were incubated at 25∘ C for 4 days in the dark, the presence or absence of fluorescence in the agar surrounding the growing colonies was determined by exposing the Petri dishes to ultraviolet light (365 nm) and expressed as positive or negative.

2.8 Statistical analysis

Data were in three independent replicates and subjected to analysis using Statistical Package for the Social Sciences (SPSS) 16.0 statistical software. Significant differences between mean values were determined using one-way ANOVA, the comparison made with Duncan's multiple range test ($P < 0.05$).

III. Results

3.1 Antifungal activity of lemongrass Eos.

Effects of doses and types of lemongrass Eos on the growth inhibition of mycelia after incubation at 25∘ C for 7 days on seven *Aspergillus* fungi were tested (figure 1, table 1). The results show that: the growth of all *Aspergillus* species tested is completely inhibited by both Eos, whatever their type at the concentration of 0.4 µl

/ml. The Eos (2) from mycorrhizal plants has completely inhibited the growth of the 7 species of fungi tested at the concentration of 0.1 µl /ml compared to the Eos (1) from non-mycorrhizal plants, where the activity was shown to increase with the concentration. At the concentration of 0.1 µl /ml, differential sensitivity of the different strains of fungi to Eos (1) compared to Eos (2) was observed.

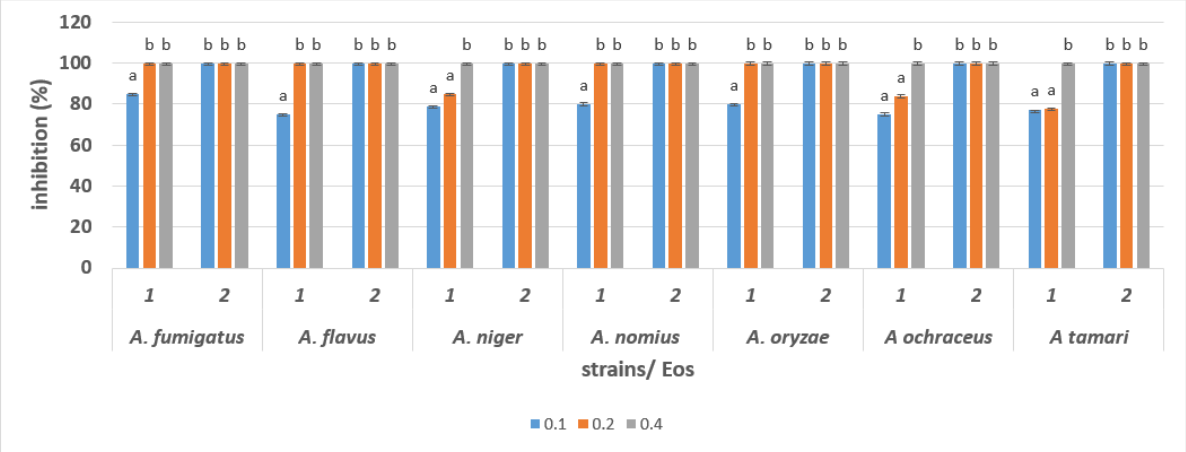
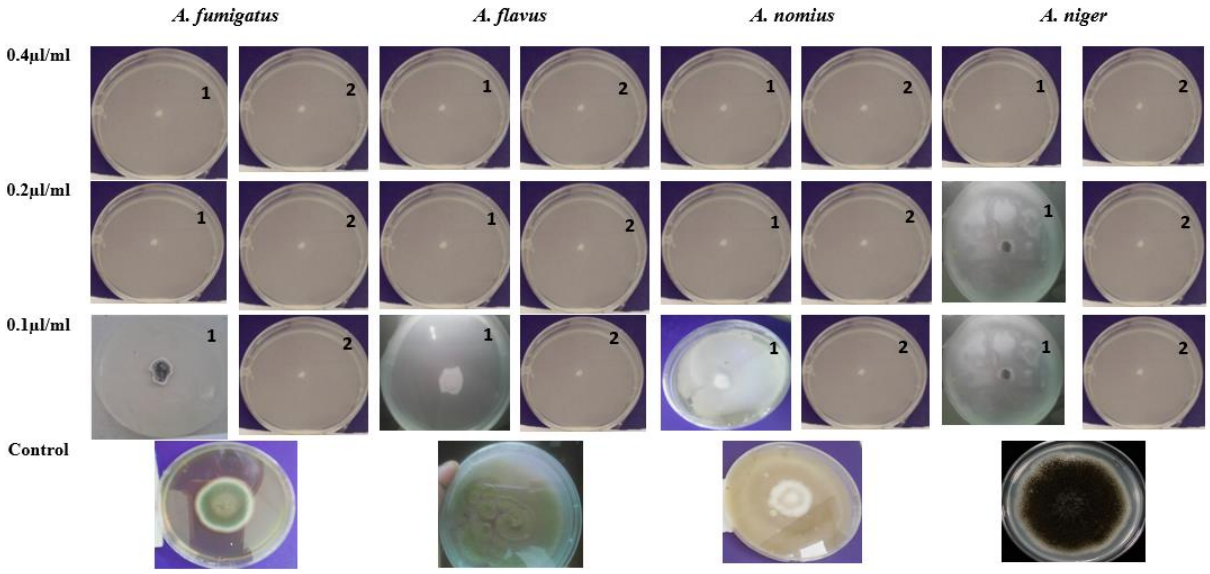


Fig 1. Influence of lemongrass Eos (1 and 2) on mycelial growth inhibition of seven *Aspergillus* fungi at 5 days of growth in PDA media.

1=Non-mycorrhizal lemongrass Eo; **2**=Mycorrhizal lemongrass Eo.
Bars with the same letter in a block with a number are not significantly different at (P<0,05)



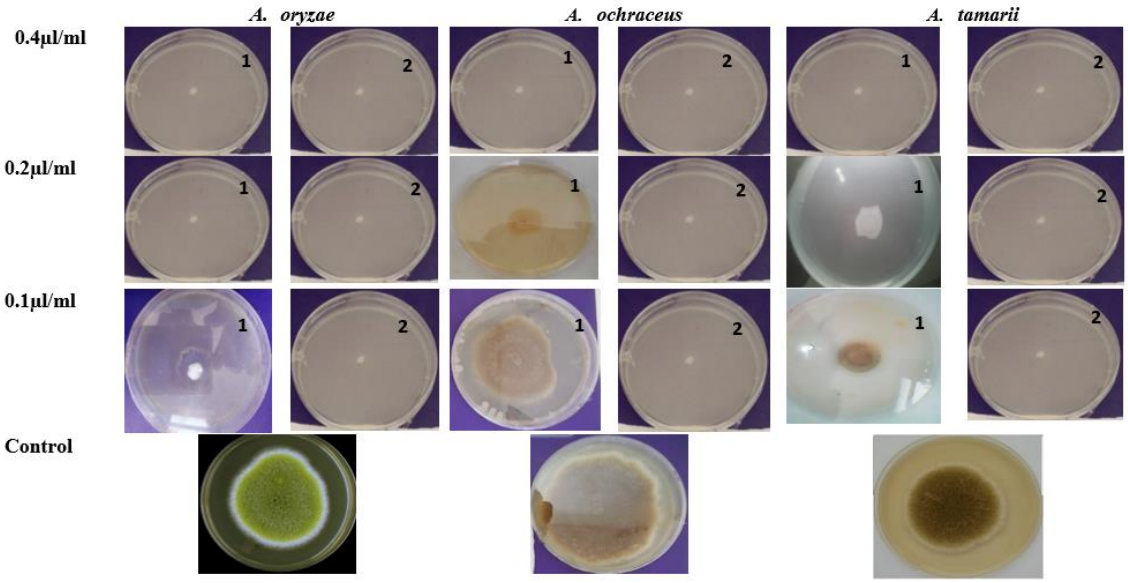


Table 1. Influence of lemongrass Eos (1 and 2) on growth inhibition of seven *Aspergillus* fungi at 5 days of growth in PDA media.
1=Non-mycorrhizal lemongrass Eo; **2**=Mycorrhizal lemongrass Eo.

3.2 Influence of lemongrass Eos on dry mycelia biomass of seven *Aspergillus* fungi.

The dry mycelial weight of seven *Aspergillus* fungi was tested for their response to different doses and types of lemongrass Eos after incubation at 25∘ C for 7 days (figure 2). Results show that regardless of the type of lemongrass Eos, no mycelium dry biomass was recorded for the 7 species of fungi studied compared to the control at the concentration of 0.4 1µl/ml. Similarly, the mycelium weight of the 7 species of fungi was also zero gram at the concentration of 0.2 µl/ml for Eos from mycorrhizal plants (2) compared to Eos from non-mycorrhizal plants (1). Although significantly sensitive to lemongrass Eos in general, the *Aspergillus* species studied are all sensitive to Eos (2), while their sensitivity to Eos (1) is different regarding the species.

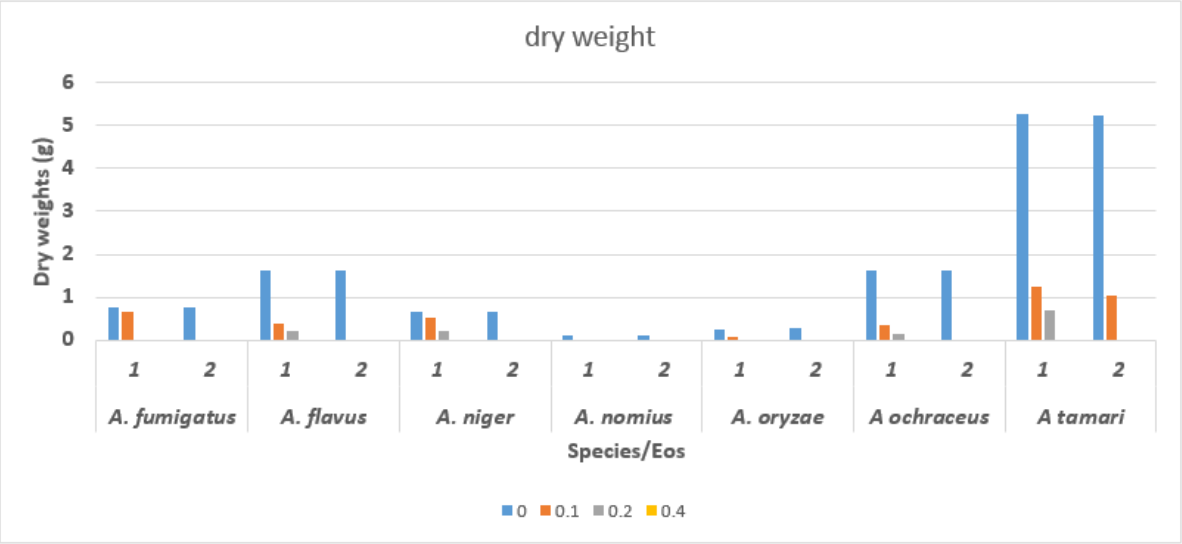


Figure 2 Influence of lemongrass Eos on mycelial dry weight of seven *Aspergillus* fungi in PDB media at 5 days of growth
(n=3). ***A***=*Aspergillus*. (1) and (2) refer respectively to non-mycorrhizal and mycorrhizal lemongrass essential oils.

3.3 Inhibition of amylase and cellulase activities by lemongrass Eos

Effects of different types of lemongrass Eos on amylase and cellulase activities after incubation at 25∘ C for 7 days on seven *Aspergillus* fungi were tested (table 2). Results show that control and test treatment did not

present any enzyme activities for all the tested fungi in regard to amylase enzymes during their growth in PDA media. However, they showed after the revelation of aureoles characteristic of cellulase enzyme activities during their growth. Those aureoles were showing to increase in the presence of Eos in the media, with the more important observed in the presence of Eo2 compared to Eo1.

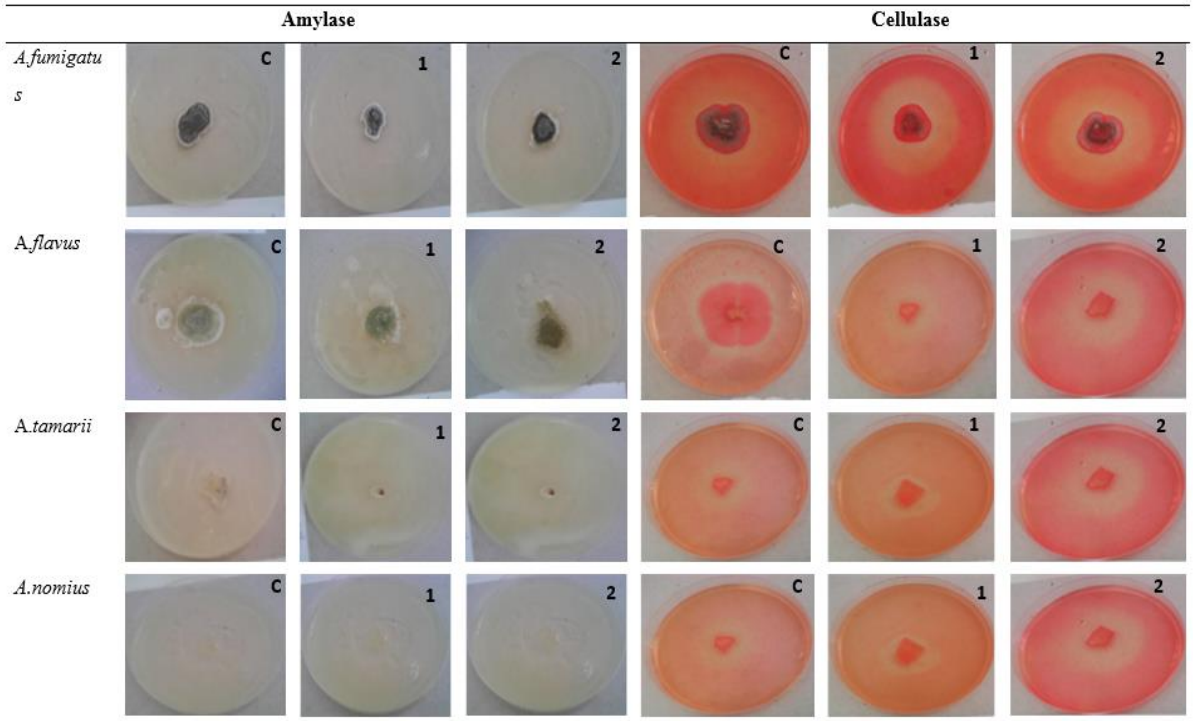


Table 2 influence of lemongrass Eos on amylase and cellulase activity of four *Aspergillus* fungi in PDB media at 5 days of growth

(n=3). (1) and (2) refer respectively to non-mycorrhizal and mycorrhizal lemongrass essential oils; C refer to control plates.

3.5 Inhibition of aflatoxin production of *Aspergillus* by lemongrass Eos

Effects of different types of lemongrass Eos on aflatoxin production of three *Aspergillus* fungi, after incubation at 25◦ C for 5 days were tested in PDA media (table 3) and CMA media (table 4). Results show that strains able to produce aflatoxins show better results on CMA media compared to PDA. Two of the three tested fungi (*Aspergillus flavus* and *Aspergillus tamari*) show blue fluorescent aureoles around their colonies in control Petri plates, given proof of the production of aflatoxins by those strains. When the media were enriched with both types of Eos, the aureoles disappear independently of the nature of the media used, proving the inhibition capacity of the extracts.

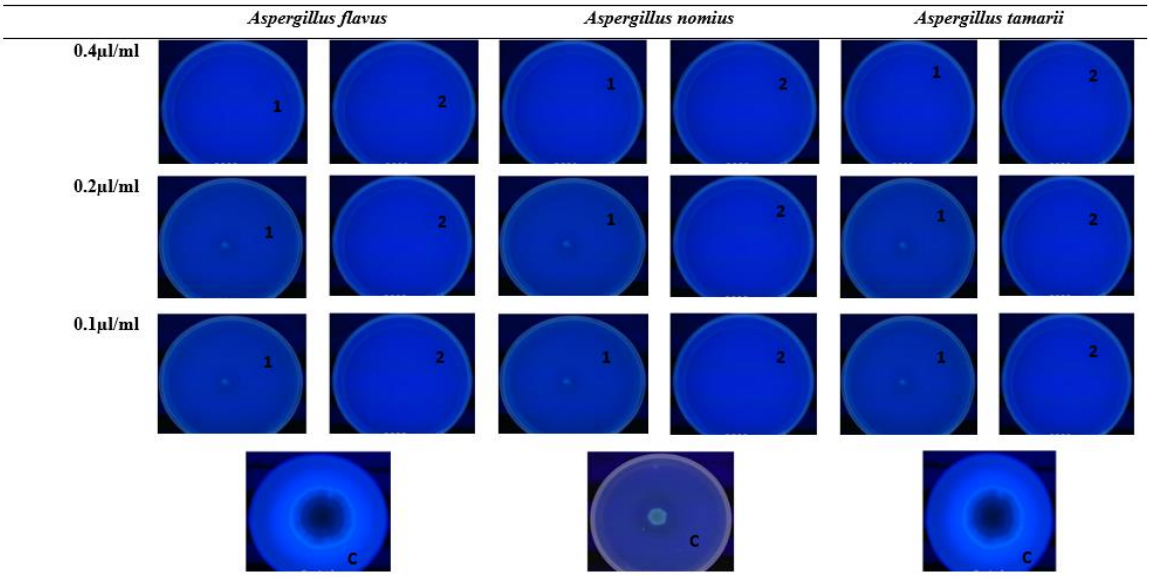


Table 3 Influence of lemongrass Eos on aflatoxins production of three *Aspergillus* fungi in PDA media at 5 days of growth

(n=3). (1) and (2) refer respectively to non-mycorrhizal and mycorrhizal lemongrass essential oils. C refer to control plates.

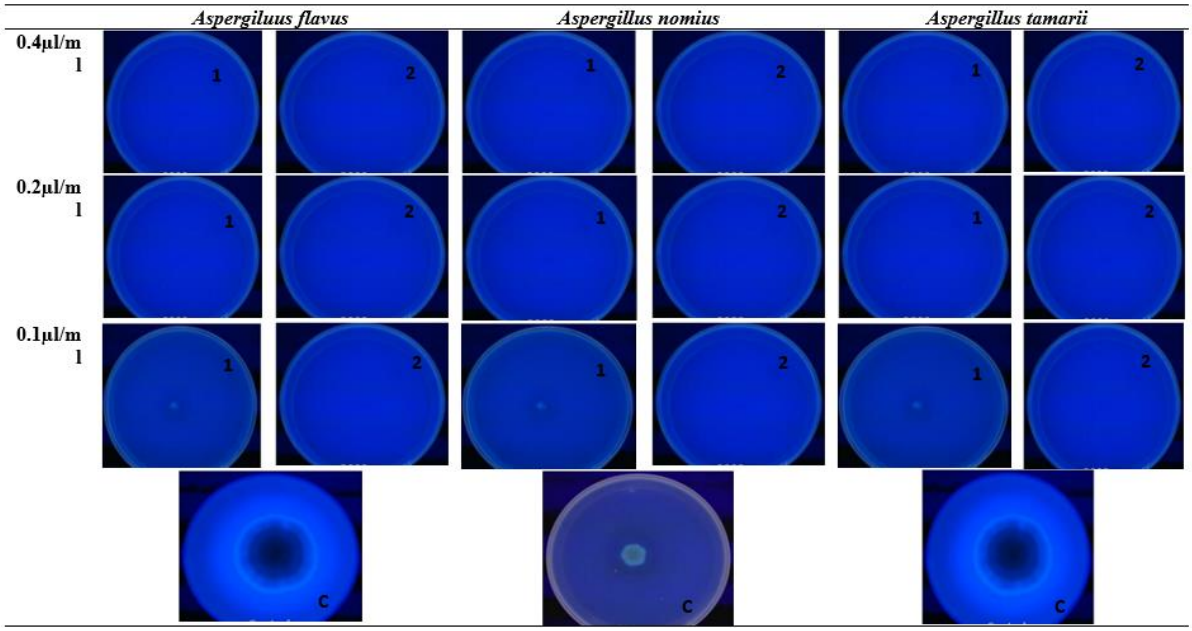


Table 4 influence of lemongrass Eos on aflatoxins production of three *Aspergillus* fungi in CMA media at 5 days of growth

(n=3). (1) and (2) refer respectively to non-mycorrhizal and mycorrhizal lemongrass essential oils.

IV. Discussion

Development of preventive strategies to overcome mold colonization and subsequence production of aflatoxins in food commodities including smoked dry fish still a challenge. These strategies range from good production and process practices to the use of biocontrol agents or natural compounds able to avoid the destruction and poisoning of food (Agriopoulou et al., 2020). Description of new natural products useful to control spoilage and toxigenic molds will be a benefit to the whole community. Moreover, studies credited with knowledge showing the inhibition effect of natural extracts in various harmful fungi impacting public health are welcome. Results from this study indicate that lemongrass Eos had anti-fungal activity against the seven *Aspergillus* species test known as *A. fumigatus, A. flavus, A. niger, A. nomius, A. oryzae, A. ochraceus and A. tamari* (figure 1, table 1). The growth inhibition capacity of the Eos was shown to vary according to the fungi species, as well as the type

of essential oil implicated. Previous studies point out the activity of essential oils in the growth inhibition of the mycelial growth of several types of fungi including *Aspergillus* (Negrelle and Gomes 2007; Chen et al., 2014; Li et al., 2020; Dong et al.,2021; Mukarram et al., 2022). Studies also noted a wide variation in growth inhibition activity, which can be a function of the origin of the essential oils used, as well as the species of fungi involved. Such observation supports the view that edaphic and growth environment conditions govern the composition and activities of plant Eos. Moreover, AM fungi which is a biofertilizer having a symbiotic relationship with plants was shown to positively impact lemongrass growth and the chemical composition of it Eos, with consequences on their antifungal properties (Fokom et al., 2019; Fokom et al., 2021). In line with the antifungal activity, it was reported that lemongrass Eos caused plasma membrane disruption and disorganization of mitochondria and resulted in Ca2+, K+, and Mg2+ leakage, with further consequences on signal transduction and fungal spore germination, destabilization of the membrane, retards of fungal spore formation and cellular respiration (Helal et al. 2007; Da Silva et al., 2008; García-Diaz et al. 2019; Moumni et al. 2021). The such metabolic impact could be perceptive on various aspects of the fungal organism. Results from this study point out the reduction of mycelia biomass production with respect to the nature of the Eos involve as well as the species of fungi (figure 2). Hence one fungi species *A tamari* shows no biomass production at all concentrations of Eo2 compared to the control. *A nomius* did not produce any biomass in the presence of both Eos at all concentrations compare to the control. The chemical composition of the lemongrass Eos will probably have a great implication in these observations. The previous study on lemongrass Eos shows that individual compounds like myrcene, linalol, neral, geraniol, geranial, and isoneral increase in concentration following AM inoculation of the plant while others are initially absent in the Eos appear when plants are inoculated (Fokom et al., 2019). Those compounds could have accounted for the activity of Eos on the growth and mycelial biomass inhibition of the tested fungi. Essential oils containing citral, a mixture of neral and geranial have shown antimicrobial, antifungal, and antiparasitic properties (Zeng et al., 2015). Moreover, studies on Linalool, a lemongrass Eos compound showed fungicidal properties and the restriction of aerial mycelia respiration (Jayasena and Jo, 2013; Boukhatem et al., 2015). Disturbances in the constitution of the cell wall as well as in the mineral balance of fungal organisms necessarily influence its metabolism, in particular the synthesis of functional molecules such as proteins and also the synthesis of macromolecules including aflatoxins. Results from this study show that: in the presence or absence of Eos, the strains of *Aspergillus* tested do not present any characteristics reflecting the activity of the enzyme amylase (table 2). Moreover, the results show that in the absence of Eos, the strains of *Aspergillus* tested present after the revelation, halos zones characteristic of cellulase activity. In the presence of Eos the diameter of these halo increases with the best diameter noted in the media containing Eo2 followed by Eo1 compared to the control (table 2). It seems possible that *Aspergillus* species do not use amylase to explore their environment during growth. However, cellulase might be a useful enzyme for fungi of this group, which help them explore their substrate for better growth. The presence of the Eos in the media leads to increases in the diameter of the halo zone around colonies, mining the extra production and activity of the enzyme. This enzyme appears to be a tool to determine the antifungal activities of plant extracts, in order to develop useful antimicrobial substances. Results of this study also show that of the three species of *Aspergillus* fungi tested, two are aflatoxin producers because a clear halo was recorded around the colonies of A flavus and A tamari (tables 3 and 4). The introduction of Eos in the media both PDA and CMA led to the disappearance of the halo zone, meaning the inhibition of the synthesis of the involved molecule by the fungi (tables 3 and 4). This observation was not a function of the type of Eo used. It seems possible that Eos creates some stress conditions that hurt the fungi metabolism and as a result, enzymes implicated in the adaptation like cellulase are more synthesized while toxic molecules like aflatoxins are not produced. Consistent with our results, other studies have also shown that EOs have the capability on inhibiting both fungal growth and metabolism with consequences on mycotoxin and enzyme production in *Aspergillus* species (Diaz et al., 2019; Bomfim et al., 2020; Da Silva et al. 2020; Lorán et al., 2022).

V. Conclusion

This study has shown that lemongrass Eos are effective agents able to inhibit mycelial growth and biomass production of Aspergillus fungi. These inhibition activities are improved when Eo are from AM fungal plant origin. Lemongrass Eos has can affect Aspergillus fungi metabolism and stimulate additional cellulase activity with the predominance of EO from AM plant origin.

Eo from this plant hurt Aspergillus metabolism by inhibiting aflatoxin production. Cellulase enzymes and aflatoxin are tools to test the antifungal activity of Eos. Lemongrass Eos are effective alternatives to the synthetic chemical for fungal control mostly harmful to consumer health and the environment.

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