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Sensitivity to *Lippia alba* (Mill.) NE Br Essential Oil of two *Aspergillus flavus* Strains Isolated from Peanut Seeds (*Arachis hypogaea* L.) Collected from Two Agroecological Zones in Senegal

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aspergillus flavus is most often associated with acute or chronic aflatoxicosis due to its ability to produce aflatoxin. These recent years, many studies have been conducted to identify biopesticides able to mitigate the aflatoxinogenic strains in soils and crops. Then, essential oils naturally contain various bioactive molecules whose antifungal properties offer promising prospects for mitigating the dangers posed by aflatoxins. This study aims to determine the chemical composition of *Lippia alba* essential oil and to assess its effectiveness on 2 strains of *A. flavus* isolated from peanut seeds in Senegal. Chemical analysis by GC-MS of the essential oil (EO) of *Lippia alba* revealed more oxygenated monoterpenes (84.3%) than hydrocarbons (8.4%) and oxygenated (2.4%) sesquiterpenes. Neral (34.6%) and geranial (46.6%) were also identified as major and distinctive biochemical components present in the essential oil extracted from *Lippia alba* plants in Senegal. Furthermore, the biological activity of this EO, at three different doses (100, 500, and 1000 ppm), was more effective in inhibiting the mycelial growth of the two isolates of *Aspergillus flavus* (TN and V), compared to Azoxystrobin (Positive control). The inhibition rates recorded with the highest dose (1000 ppm) reached 91.4% on the TN isolate (Peanut Basin isolate) and 84.6% on the V isolate (Casamance isolate). In contrast, after 11 days of incubation, Azoxystrobin at 1000 ppm produced 34.3% and 66.9% inhibition rates, respectively, on the TN and V isolates. Due to their chemical composition, essential oils are positioned as an alternative to synthetic pesticides and in the fight against crop pests and mycotoxins. However, for the practical use of this EO as a fungicide, future research would need to include searching for a suitable carrier appropriate inert material, allowing the development of a biopesticide formula directly applicable to peanut seeds without any negative impact on their physico-chemical, functional, and organoleptic properties.

Keywords: Essential oils; Aspergillus flavus; antifungal activity; aflatoxin; peanuts; miller.

1. INTRODUCTION

Lippia alba (Miller) N.E. Brown is an aromatic plant belonging to the Verbenaceae family. It mainly grows in South and Central American countries as well as in tropical African regions. "*Lippia alba* (Mill.) N. E. Brown is a plant mainly used in the form of infusions and decoctions of its leaves due to their sedative action. The pharmacological properties of *L. alba* as an anxiolytic, sedative and motor relaxant in rats started to draw attention as a possible anaesthetic for fish" (Kampke et al., 2018; Malik et al., 2021). Traditionally, plants of this species are used in various preparations as remedies for gastrointestinal and respiratory ailments, antimalarial, and antiviral treatments (Caballero-Gallardo et al., 2022, Glamočlija et al., 2011, Quintero et al., 2021). The leaves are sometimes used in food preservation (Mutlu-Ingok et al., 2020). These observations have led to several studies conducted on *L. alba*, which have highlighted interesting biological properties of its essential oil (EO), attributable to its various chemical constituents or extracts (Hennebelle et al., 2006, Mesa-Arango et al., 2009, Sales et al., 2022). "The results for the composition of *L. alba* essential oils are invariably non-coincident, as has been seen in the literature. The distinct results described for the composition of *L. alba* oil may be associated with different environmental conditions for the plant production" (da Silva Junior et al., 2019, de Albuquerque Lima et al., 2021). Indeed, the EO of *L. alba* exhibits different chemical chemotypes related to the part of the plant from which it is extracted and the specific characteristics of its origin area (Hennebelle et al., 2006, Mesa-Arango et al., 2009, Santos Filho et al., 2023). "Terpenic compounds (limonene, carvone, citral, β-caryophyllene, tagetenone, myrcene, γ-terpinene, camphor, 1,8-cineole, and estragole) are frequently identified in this essential oil composition" (Santos Filho et al., 2023, Shukla et al., 2009 Hennebelle et al., 2006, Joulain, 1998, Adams et al., 2007). The antimicrobial activity of *L. alba* has been demonstrated against both human and plant fungal diseases. For example, the citral and myrcene-citral chemotypes showed a significant impact against fungal pathogens (*Candida albicans*, *Trichophyton rubrum*, and *Fonsecaea pedrosoi*) (Sales et al., 2022, NIST, 2008). Furthermore, antifungal and anti-aflatoxin activities against *Aspergillus flavus* and *Aspergillus parasiticus* have been demonstrated, even at low treatment doses, with the citral and myrcene-citral chemotypes (Tang et al., 2018, Mesa-Arango et al., 2009), as well as with the neral and geranial chemotypes (Tang et al., 2018, Pandey et al., 2016). This led us to carry

out further investigations about the chemical components of the essential oil extracted from *L. alba* leaves collected in Senegal, and to test its efficacy against two *A. flavus* strains isolated from peanut seeds collected in two agroecological zones in this country.

2. MATERIALS AND METHODS

2.1 *A. flavus* strains

The *Aspergillus flavus* isolates used in this study were obtained at the Phytopathology and Weed Science Laboratory of the Directorate of Plant Protection (DPV) of Senegal, from peanut seeds collected in Taïba Niassène (isolate TN) and Velingara (isolate V) (Fig. 1).

On AFPA, V isolate appears fluffy white, producing many small black sclerotia whose density varies from the centre to the periphery of the colony (Plate 1). Isolate TN appears without sclerotia, with a powdery texture and a yellow colour that becomes light green at the colony edges (Plate 2). The growth rate of the TN isolate is faster than that V isolate, with an average diameter of 9 cm after 7 days of incubation. On the reverse side, both isolates are characterised by brown pigmentation, which is, however, more pronounced in the TN isolate (Plate 2). Isolate V is rather characterised by a radiating structure on the reverse side (Plate 1). Under the microscope, both isolates present a hyaline, non-septate conidiophore topped with uniseriate conidial heads producing globular and clear-looking conidia (Plates 1, 2).

2.2 Plant Material

The essential oil was extracted from *Lippia alba* leaves collected in Sindia (Thiès, Senegal) (Fig. 2). The identification of the plant species was carried out at the Laboratory of the Fundamental Institute of Black Africa (IFAN) of Cheikh Anta Diop University of Dakar. “The leaves were air-dried for two weeks at room temperature and then subjected to hydrodistillation for 5 hours using a Clevenger-type apparatus according to the method recommended in the European Pharmacopoeia” (Gomes et al., 2019). The yield of essential oil obtained from the extraction was 1.7%.

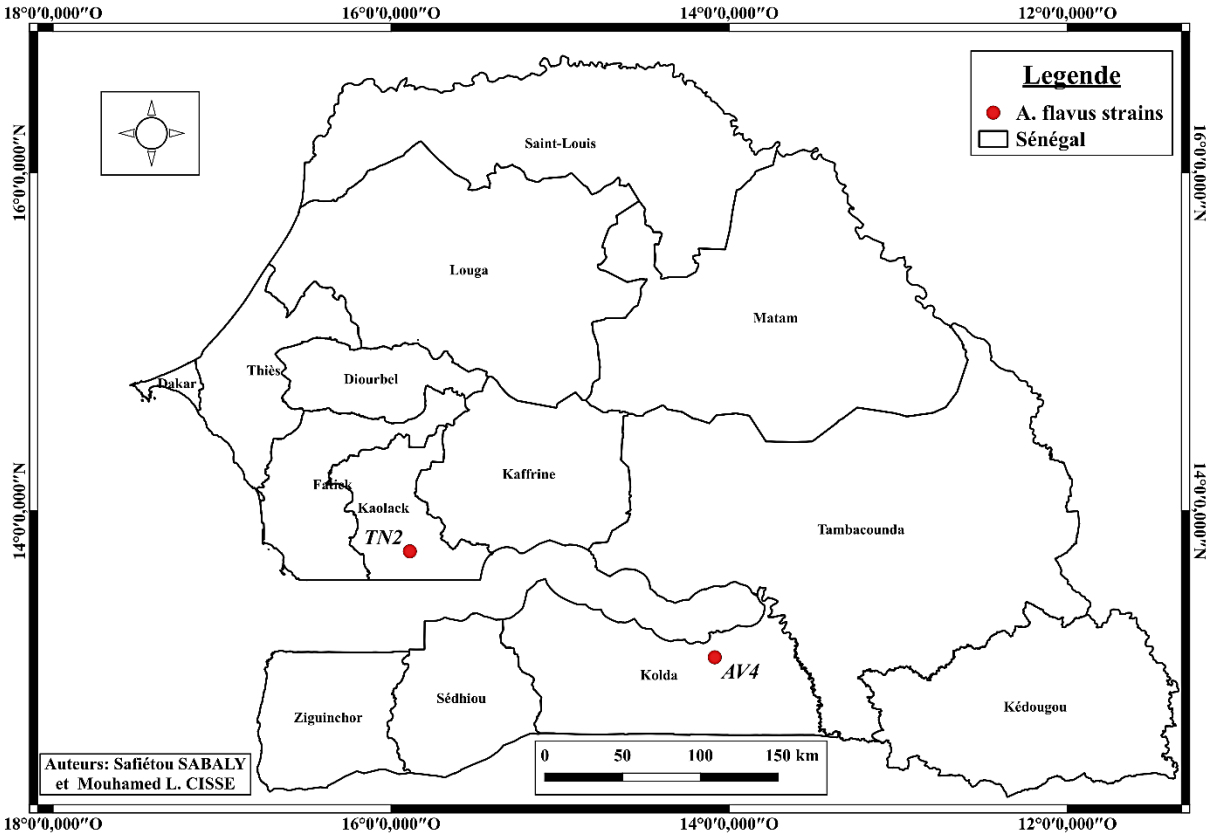

Fig. 1. Peanut samples collection sites

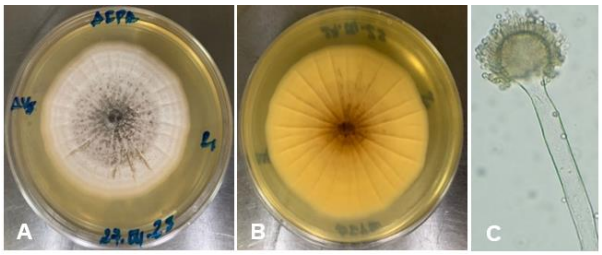

Plate 1. Macroscopic and microscopic aspects of the A. flavus V strain

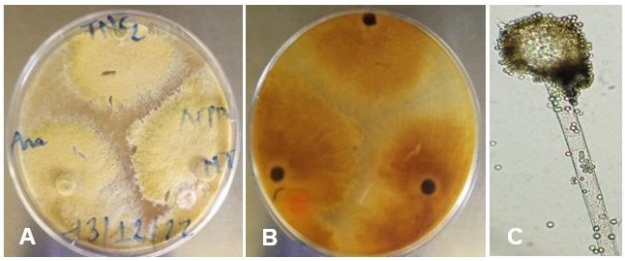

Plate 2. Macroscopic and microscopic aspects of the A. flavus TN strain

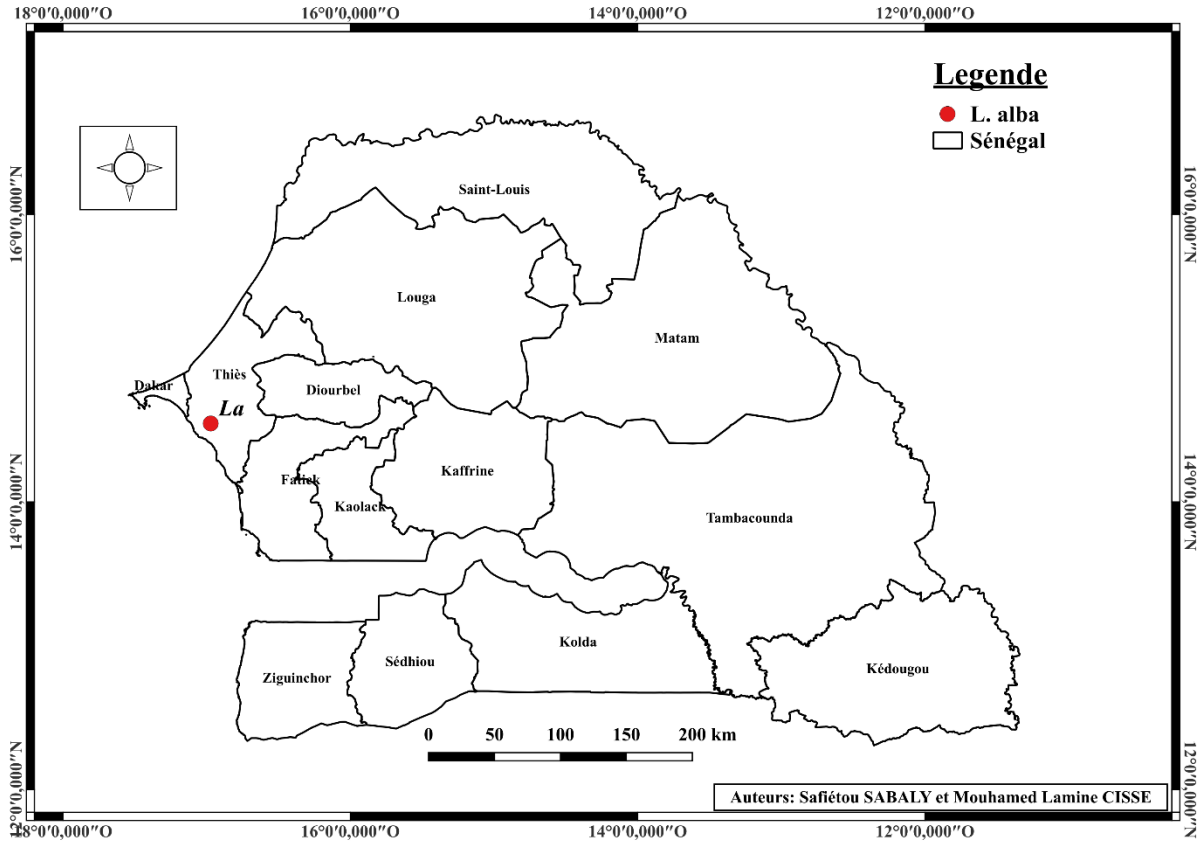

Fig. 2. *L. alba* plant harvesting sites

2.3 Chemical Composition

The chromatographic analyses were carried out using a Perkin-Elmer Autosystem XL GC apparatus (Waltham, MA, USA) equipped with a dual flame ionisation detection (FID) system and fused-silica capillary columns, namely Rtx-1 (polydimethylsiloxane) and Rtx-wax (poly-

ethyleneglycol) (60 m × 0.22 mm i.d; film thickness 0.25 µm). The oven temperature was programmed from 60 to 230 ◦C at 2 ◦C/min and then held isothermally at 230 ◦C for 35 min: hydrogen was used as carrier gas (1 mL/min). The injector and detector temperatures were maintained at 280 ◦C, and samples were injected (0.2 µL of pure oil) in the split mode (1:50). The retention indices (RI) of the compounds were determined proportionally to the retention times of a series of n-alkanes (C5-C30) by linear interpolation using the equation of Van den Dool and Kratz (1963) via Perkin-Elmer software (TotalChrom navigator). "The relative proportions of the oil constituents were determined from the GC peak areas, without the use of correction factors. In addition, the samples were analyzed using a Perkin-Elmer Turbo mass detector (quadrupole) coupled with a Perkin-Elmer Autosystem XL, equipped with Rtx-1 and Rtx-Wax fused silica capillary columns. The oven temperature was programmed from 60 to 230°C at 2°C/min, then maintained isothermally at 230°C (35 min): hydrogen was used as the carrier gas (1 mL/min). The following chromatographic conditions were used: injection volume, 0.2 µL of pure oil; injector temperature, 280°C; fractionation, 1:80; ion source temperature, 150°C; ionization energy, 70 eV; MS(EI) acquired over the mass range 35-350 Da; scan speed, 1 s. The identification of the different components was based on (a) comparison of their GC retention indices (RI) on non-polar and polar columns, determined from the retention times of a series of n-alkanes with linear interpolation, with those of authentic compounds or data from the literature; (b) computer comparison with commercial mass spectrum libraries and comparison of the spectra with those in our personal library; and (c) comparison of RI and MS spectral data from authentic compounds or data from the literature" (Hennebelle et al., 2006, Defat, 2023, García-Barriga, 1974).

2.4 Treatments and Incubation

The culture medium, based on potato dextrose agar (PDA), was prepared by dissolving 39 g of powder in 1 L of distilled water, then sterilizing at 121°C for 20 minutes. A volume of 1 ml of essential oil (EO) was taken and mixed with 1 ml of pure ethanol to facilitate its dissolution. The EO + ethanol mixture was then added at different concentrations (100, 500, and 1000 ppm) to 50 ml of PDA cooled to 50°C. The resulting solution was homogenized for 1 minute and distributed into three 9 cm diameter Petri dishes. Azoxystrobin at a dose of 1000 ppm and PDA + ethanol were used as positive and negative controls, respectively (Table 1). Discs 0.6 cm in diameter were taken from each fungal strain, aged 5 days on PDA, and placed in the center of the dishes containing the different culture media. All inoculated media were then placed in an incubator set at 25°C. In each Petri dish, the mycelial growth of *A. flavus* was monitored through daily measurements of the colony diameter using a graduated ruler. After 11 days of culture, the inhibition rate (IR) of the mycelial growth was calculated using the following formula:

$$\mathrm{IR}(\%) = \frac{\mathrm{DT0} - \mathrm{DT}}{\mathrm{DT0}}$$

DT0 = colony diameter in the negative control; DT = colony diameter in the treated medium.

2.5 Statistical Analyses

The data for this study were stored in an Excel spreadsheet, which was also used for the graphical representations. Statistical analyses (analysis of variance, and the Student Newman-Keuls comparison of means) relating to the inhibition rate corresponding to the different treatment were performed at 5% threshold.

Table 1. Overview of the tested treatments

Traitement code	Isolates	Product	Dose (ppm)	Study Status
T0		PDA	-	Negative control
T1			100	
T2	TN	EO *L. alba*	500	Tested
T3			1000	
T4		Azoxystrobin	1000	Positive control
T0'		PDA	-	Negative control
T1'			100	
T2'	V	*EO L. alba*	500	Tested
T3'			1000	
T4'		Azoxystrobin	1000	Positive control

3. RESULTS AND DISCUSSION

3.1 Chemical Composition of the Essential Oil

The essential oil yield obtained from the *L. alba* leaves was 1.7%. The chemical analysis of the essential oil reveals, quantitatively, 19 constituents (Table 2) representing qualitatively 96.4% of the total composition. These results showed mostly a citral chemotype composed of 46.6% geranial and 34.6% neral. Minor compounds were recorded in small proportions: trans-β-caryophyllene (4.2%), caryophyllene oxide (2%), isogeranial (1.2%), and limonene (0.7%) (Table 2).

This similar high proportion of citral was observed in several essential oils extracted from this species growing in ecologically different areas. Indeed, with a yield of 0.15%, the essential oil extracted from *L. alba* leaves growing in Brazil contained 33.32% neral and50.94% geranial according to Glamočlija et al., (2011). Similarly, high citral levels (60%) were found in essential oils extracted from the aerial parts of this plant harvested in autumn, growing in Argentina (Hennebelle et al., 2006), and in winter in Brazil with some amounts of geraniol (9%) and myrcene (5%) (Tavares et al., 2005). In Côte d'Ivoire, the plant predominantly showed citral (45.86%) and geraniol (14%) (Coulibaly et al., 2023). However, other chemotype variants are obtained elsewhere. The γ-Terpinene

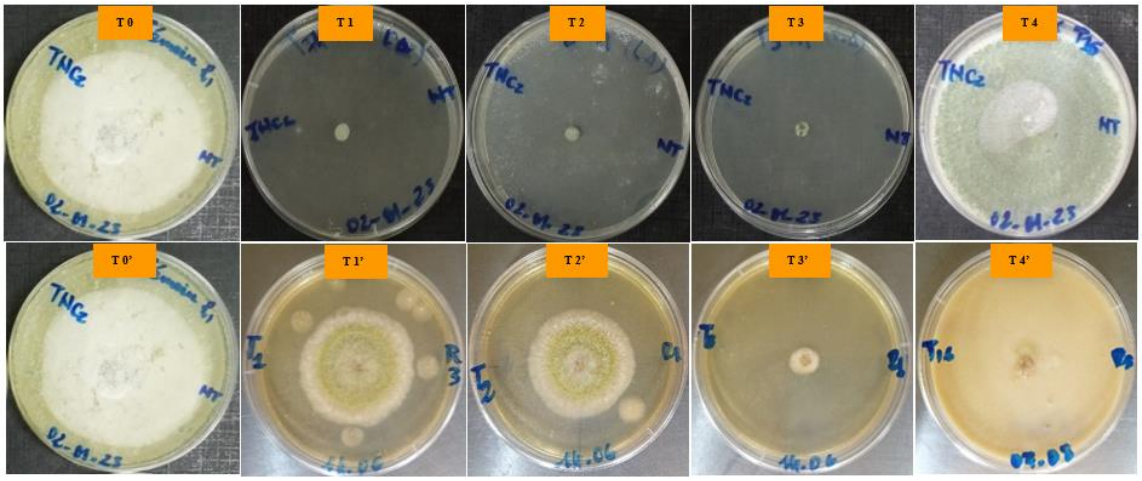

Plate 3. Showing the different treatments impact on the mycelial growth of the V and TN strains

Table 2. Chemical composition of the *L. alba EO*

N[a]	Compounds	lRI[b]	RIa[c]	RIp[d]	*L. alba*
1	6-methylhept-5-en-2-one	963	963	1337	0.5
2	Limonene	1025	1022	1200	0.7
3	(*E*)-β-Ocimene	1041	1034	1247	0.1
4	Linalol	1086	1081	1544	0.5
5	Citronellal	1132	1131	1479	0.1
6	Isogeranial	1156	1159	1748	1.2
7	Neral	1215	1214	1679	34.6
8	Geranial	1244	1247	1731	46.6
9	Geranyl acetate	1362	1361	1752	1.3
10	*β*-Elemene	1389	1386	1589	0.5
11	Trans-β-Caryophyllene	1421	1417	1583	4.2
12	α-Guaiene	1440	1440	1583	0.9
13	Humulene	1455	1450	1660	1.1
14	*γ*-Muurolene	1474	1470	1681	0.4
15	Germacrene D	1479	1476	1704	0.7
16	*α*-Bulnesene	1503	1494	1711	0.5

N[a]	Compounds	IRI[b]	RIa[c]	RIp[d]	*L. alba*
17	Trans-α-bisabolene	1530	1532	1753	0.1
18	Caryophyllene oxide	1570	1573	1959	2.0
19	Humulene epoxyde 2	1602	1598	2044	0.4
	Hydrocarbon monoterpenes				0.8
	Oxygenated monoterpenes				84.3
	Hydrocarbon sesquiterpenes				8.4
	Oxygenated sesquiterpenes				2.4
	Other compounds				0.5
	Total identified (%)				96.4
	Yields (w/w vs dry material)				1.7

[a] Order of elution is given on apolar column (Rtx-1).
[c] Retention indices on the apolar Rtx-1 column (RIa).
[d] Retention indices on the polar Rtx-Wax column (RIp).

chemotype (46%) with traces of p-cymene (9%) and β-caryophyllene (7%) have been reported from Brazil (Gomes et al., 2019). A high concentration of Limonene (34%-47%), piperitone (37%-24%), and 1,8-cineole (10%-13%) was obtained from leaves harvested in autumn in Argentina (Hennebelle et al., 2006). The essential oil obtained from *Lippia alba* leaves harvested during the fresh dry season in the commune of Niague-Tivaoune Peulh in Senegal was composed of almost equivalent proportions of limonene (37.5%) and citral (39.9%) (Defat, 2023).

3.2 Antifungal activity of *Lippia alba* EO

The results about mycelial growth inhibition revealed different sensitivity levels of the two *A. flavus* isolates to the *L. alba* EO, higher compared to Azoxystrobin. Consequently, the action of the EO was more pronounced on the TN isolate than on the V isolate, proportionally to the application doses (Plate 3). The statiscal analysis shows a highly significant difference ($p < 0.000$) between the 2 strains' radial growth inhibition rates. Indeed, with the low dose (T1 and T1'), the inhibition rate was 70.5% for the V isolate, whereas it reached 88.8% for the TN isolate. The same trend has been observed with the intermediate dose (T2 and T2'), in which were established at 80.2% and 91.4% the inhibition rates for the isolates V and TN, respectively. Similarly, the inhibition rate was stabilised at 91.4% for the TN isolate and increased for the V isolate up to 84.6% in the maximum dose of 1000 ppm (T3 and T3'). Furthermore, on the negative controls (T0 and T0'), they showed a rapid mycelial growth, covering the entire surface of the Petri dishes within 72 hours. On the positive controls T4 and T4' (Azoxystrobin), the recorded inhibition rates were respectively 34.3% and 66.9% for the V and TN isolates, after 11 days of incubation.

However, the radial growth inhibition showed a decreasing evolution in time, for all the treatments, except for the T2' and T3' (V on 500 & 1000 ppm EO) during 11 days of incubation. This decrease was found to be more pronounced for the TN strain than for the V one. Then, at the lowest dose T1 (100 ppm), the inhibition rate has fallen from 70.5% to 36.1% after 11 days of incubation. For the treatments T2, T3, and T4, the inhibition rates were stabilised at 47.47% at the 11th day. However, with the TN strain, the decrease began after 10 dayswith the lowest dose T1' (100 ppm) and reached 73.7% at the 11th day (Fig. 3B).

The bioactive properties of *L. alba* EO and its components have long been used for pharmacological, medicinal, aromatic, or cosmetic purposes (García-Barriga, 1974, Meskaoui et al., 2008, Cormier-Salem & Roussel, 2009 Festy, 2014, Bersan et al., 2014, Sissinto Adjovi et al., 2022). Furthermore, many studies have shown biocidal effects of *L. alba* EO against stored-product insects (Caballero-Gallardo et al., 2022), as well as against pathogenic microorganisms, bacteria (*Escherichia coli, Listeria innocua, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella choleraesuis and Staphylococcus aureus*) and fungi (*Aspergillus flavus, A. glaucus, A. ochraceous, Colletotrichum gloeosporioides, A. niger, C. musae,* and *Fusarium oxysporum*) (Glamočlija et al., 2011, Mesa-Arango et al., 2009, Sales et al., 2022, Escobar et al., 2010). Antifungal test results showed that *L. alba* EO possesses strong antifungal properties, particularly against *Aspergillus flavus*. It significantly reduces mycelial growth, with a much more remarkable effect on the TN isolate compared to the V isolate. This differential

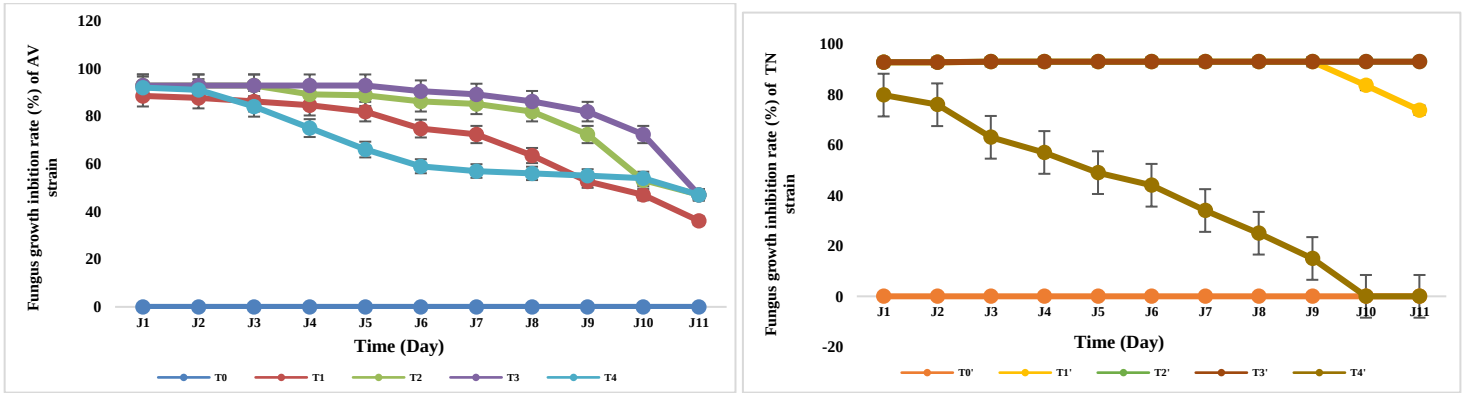

Fig. 3. Evolutionary tendencies of the v (A) & TN (B) A. flavus strains growth inhibition by *L. alba* EO during 11 days incubation

Table 3. Variation of the mycelial growth inhibition rates of the *A. flavus* V and TN strains depending to the media treatments

Doses (ppm)	AV isolate		TN isolate	
	Inhibition Rate (%)[1]	Average Diameter (cm)[1]	Inhibition Rate (%)[1]	Average Diameter (cm)[1]
100 (T1, T1')	70,5±18,2	3.9±0.02	88,8±7,4	2,1±0.02
500 (T2, T2')	80,2±16,2	2.2±0.001	91,4±5	0.8±0.001
1000 (T3 et T3')	84,6±14	2,7±0.22	91,4±5	0.8±0.001
1000 (Azoxystrobin)	66,9±15,9	4.2±0.22	34,3±16	8.20±0.22

response may be due to the intrinsic properties of the microorganisms being tested on one hand, and the biological properties of the constituents of *L. alba* EO on the other. Composed mainly of Neral (34.6%) and Geranial (46.6%), *L. alba* EO has a remarkable effect against pathogenic microorganisms, especially fungi (Mutlu-Ingok et al., 2020, Santos Filho et al., 2023, Glamočlija et al., 2011). These results are consistent with those of Pandey et al. (2016), who studied the efficacy of certain essential oils against the proliferation of *Aspergillus flavus* with a particular reference to the efficacy of *L. alba* EO. Likewise, Shukla et al. (2009) demonstrated the effectiveness of *L. alba* (Mill.) NE Brown EO and its constituents against fungi isolated from certain edible legume seeds and the production of aflatoxin B1. According to their findings, geraniol (22.2%) and nerol (14.2%), as major components, showed remarkable antifungal effects against all fungal isolates with a low application dose of 1 µL/mL. Similarly, Pandey et al. (2016) showed that "the application of a *L. alba* EO rich in geranial (36.9%) and neral (29.3%), and to a lesser extent in myrcene (18.6%) in the storage system, significantly inhibited fungal proliferation and aflatoxin production without affecting the seed germination rate". A strong effectiveness of *L. alba* EO against Gram-positive and Gram-negative bacterial species was observed with the following concentrations: 50%, 25%, 6.25%, 3%, 1.5%, 0.8%, 0.4%, and 0.2%) (Pandey et al., 2016). Due to its heterogeneous composition and the antimicrobial activity of most of its components, it seems unlikely that there is a single mechanism of action or that a single constituent is responsible for this high antimicrobial activity (Porfírio et al., 2017, Carson et al., 2002, Oussalah, 2006). Indeed, according to Guinoiseau (2010) and Caballero-Gallardo et al. (2022) limonene is a component of *L. alba* EO with an isopropyl group at position 4 that exhibits remarkable biological activity. Additionally, the synergistic effect of caryophyllene oxide on the efficacy of this EO has been reported by Gimenes et al. (2021), (Sá Filho et al., 2022). Furthermore, the sensitivity between the two isolates to *L. alba* EO could explain this observed difference in efficacy. Indeed, the membrane properties of microorganisms appear to provide some resistance to toxic agents (Guinoiseau, 2010). However, this barrier shows some vulnerability with the increase in the application dose of the treatment product, as demonstrated by Escobar et al. (2010) and Gimenes et al. (2021). Several studies indicate that the toxicity of EO toward microorganisms is associated with the lipophilic nature and low molecular weight of its constituents. This allows the EO to quickly cross cell membranes, causing changes in their conformation and functions, thereby increasing their permeability (Oussalah, 2006, Bhavaniramya et al., 2019). Furthermore, the particularity between the two isolates is mainly characterised by the production of sclerotia by V strain, which could justify the reduced effectiveness of different doses of *L. alba* EO on the latter. Indeed, the secretion of hydrolytic enzymes (Karimi-Avargani et al., 2020, Mellon et al., 2007, Ningthoujam et al., 2022) and the production of sclerotia (survival structures) (Barros et al., 2005, Senghor et al., 2021, Geiser et al., 2000) thus confer certain strains of *Aspergillus flavus* high virulence and resistance. Furthermore, the relationship between aflatoxin production, the sclerotial phenotype, and the degree of resistance has been reported by Escobar et al. (2010) and Gao et al. (2007).

4. CONCLUSION

The study showed that the *L. alba* EO (geranial 46.6% and neral 34.6%) has a high antifungal activity against both isolates of *Aspergillus flavus*. However, it appears to be less effective on the isolate from Casamance (V) than on the one from the Peanut Basin (TN), showing respectively 84.6% and 91.4% of mycelial growth inhibition rates on the highest dose (1000 ppm) tested. Thus, *L. alba* EO could be used safely to protect peanuts against the fungus *A. flavus*. The

use of essential oils is a promising method, avoiding synthetic chemical fungicides and offering a new approach to managing mycotoxin-producing fungi. However, for the practical use of this EO as a fungicide, future research would need to include searching for a suitable carrier appropriate inert material, allowing the development of a biopesticide formula directly applicable to peanut seeds without any negative impact on their physico-chemical, functional, and organoleptic properties.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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