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Research Article

**Fungal Communities Associated with *Manihot esculenta* and the
In vitro Antifungal Efficacy of a Gelatin-Peppermint Oil
Formulation**

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ABSTRACT

This study investigates the fungal diversity associated with *Manihot esculenta* (cassava) and evaluates the antifungal efficacy of a gelatin-peppermint oil formulation against the isolated fungi. Fungal samples were collected from various locations in Lagos, Nigeria, over five months, from the phylloplane, phyllosphere, rhizoplane, and rhizosphere habitats. Six fungal species were identified, with *Aspergillus flavus*, *Aspergillus niger*, *Rhizopus stolonifer*, *Penicillium* sp., *Colletotrichum* sp., and *Macrophomina phaseolina* being the most prevalent. Diversity analysis revealed that the rhizoplane harboured the highest species evenness ($H' = 1.3708$, $D = 0.7427$), while the rhizosphere exhibited the least diversity ($H' = 1.0872$, $D = 0.6593$). Pathogenicity tests confirmed that *Colletotrichum* sp. and *Macrophomina phaseolina* caused typical fungal symptoms, including chlorosis and necrotic lesions. The in vitro antifungal assessment demonstrated statistically significant inhibition of mycelial growth across all tested species (two-way ANOVA, $p < 0.0001$ in all cases; Tukey's HSD post hoc test). For *Aspergillus flavus*, the control group showed 4.15 cm of radial mycelial growth, whereas treatment with 10% peppermint oil in gelatin resulted in only 0.65 cm of growth (84.3% inhibition). Similarly, *Rhizopus stolonifer* exhibited 4.16 cm of radial growth in the control group, while the 10% peppermint oil formulation limited growth to 0.28 cm (93.3% inhibition). These results confirm the potential of gelatin-based peppermint oil formulations as effective biocontrol agents for managing cassava fungal diseases, while also highlighting the ecological variation in fungal diversity across cassava-associated habitats. This approach offers a sustainable alternative to chemical fungicides.

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INTRODUCTION

Manihot esculenta Crantz, commonly known as cassava, is a vital subsistence crop for

populations in tropical and subtropical regions and contributes substantially to global food security. As a carbohydrate-rich staple, this

woody shrub meets both nutritional requirements and economic stability for agricultural communities throughout sub-Saharan Africa, South and Southeast Asia, and Latin America (McCallum *et al.*, 2017). While cassava demonstrates notable tolerance to abiotic stresses, including drought, low soil fertility, and marginal growing conditions, the crop remains vulnerable to significant biotic constraints, particularly fungal phytopathogens that compromise yield and quality. The microbial communities around cassava habitats have both positive and negative effects on cassava growth, with implications for the surrounding environment. Phyllosphere and phylloplane fungi, which live on the surface and surrounding areas of leaves, threaten cassava productivity by causing diseases such as Cassava Anthracnose (*Colletotrichum gloeosporioides*), thereby compromising crop quality (de Oliveira *et al.*, 2020). Similarly, root-associated fungi in the rhizosphere and rhizoplane, which thrive on the root surface and in the surrounding environment, can cause root rot and other diseases, further reduce yield and impact plant health (Olunoiki and Ayansina, 2021). Together, these fungal communities interact in complex ways with cassava, affecting both plant health and the broader ecosystem. Beneath the soil, the rhizosphere and rhizoplane are inhabited by diverse microbial communities, including fungi, which interact closely with the plant roots. The rhizosphere, the region of soil influenced by root activity, is rich in microbial life that affects nutrient uptake, soil structure, and plant health. Among the beneficial fungi found in this region are arbuscular mycorrhizal fungi (AMF), which enhance nutrient uptake, particularly phosphorus, and help plants withstand abiotic stresses such as drought (Berg and Smalla, 2009). The rhizoplane, the region of the root surface directly influenced by soil microorganisms, also harbours a range of fungi that can benefit plant health by improving root structure and promoting nutrient uptake. However, under conditions of plant stress or soil disturbance, some fungi in the rhizoplane may become pathogenic, leading to root rot and other detrimental effects that reduce plant yield and health (Ojo-Omoniyi *et al.*, 2025).

Together, the phylloplane and phyllosphere, alongside the rhizoplane and rhizosphere, form interconnected microbial ecosystems that play critical roles in supporting plant growth and resilience. The health of these microbial communities, influenced by environmental factors and plant stress, determines not only the plant's ability to absorb nutrients but also its overall resistance to pathogens, contributing to both plant health and broader ecosystem functioning (Kumar *et al.*, 2024).

Despite the essential roles fungi play in the cassava plant microbiome, the proliferation of pathogenic species can significantly compromise cassava's productivity and quality. Traditional methods of managing these pathogens have relied heavily on chemical fungicides. While effective, the extensive use of chemical agents has led to several environmental and health concerns, including water contamination, harm to non-target organisms, and the emergence of resistant fungal strains (Zubrod *et al.*, 2019). As such, there is a growing need for alternative, sustainable disease management strategies that reduce reliance on harmful chemicals and promote ecological balance.

Biological control has emerged as a promising solution to these challenges. This approach uses natural organisms or compounds to suppress pest populations and manage plant diseases, offering a more sustainable alternative to chemical pesticides (Aria *et al.*, 2025). One innovative biological control method involves the integration of gelatin and peppermint oil. Gelatin, a biodegradable and non-toxic biopolymer derived from collagen, serves as an effective carrier for bioactive compounds, enhancing their stability and delivery (Aitboulahsen *et al.*, 2018; Aria *et al.*, 2025). Peppermint oil, rich in menthol, has demonstrated antifungal properties by disrupting fungal cell membranes and inhibiting the growth of a broad range of plant pathogens (Edris and Farrag, 2003). When combined, gelatin and peppermint oil form a synergistic biocontrol formulation that could be used to manage fungal diseases associated with cassava communities (Aitboulahsen *et al.*, 2018; Edris and Farrag, 2003; Helal, 2017).

This study seeks to investigate the effects of a gelatin-peppermint oil formulation on the fungal

communities associated with cassava leaves and roots, including the phylloplane, phyllosphere, rhizoplane, and rhizosphere. We hypothesised that incorporating peppermint oil into a gelatin matrix would significantly inhibit the mycelial growth of pathogenic fungi isolated from cassava habitats compared to untreated controls.

MATERIALS AND METHODS

Sample Collection

The study was conducted across five distinct locations in Lagos, Nigeria, to collect samples from mature *Manihot esculenta* (cassava) plants. The locations were Agura (Ikorodu), Ijede (Ikorodu), Imota (Ikorodu), National Stadium (Surulere), and the University of Lagos (Akoka), with geographical coordinates of 6°36'44"N, 3°34'55"E; 6°34'0"N, 3°35'18"E; 6°39'49"N, 3°40'11"E; 6°29'49"N, 3°21'53"E; and 6°31'5"N, 3°23'10"E, respectively.

The sampling procedure was conducted over a five-month period, with collections occurring once a month. During each collection event, leaf, soil, and aero-samples from both leaf and root surfaces were collected using sterile aluminium foil, zip-lock bags, and gloves. These tools were utilized to maintain contamination-free handling of the samples. Immediately after collection, the samples were transported to the laboratory at 4°C under refrigerated conditions to mitigate the risk of degradation. All samples were cultured within 24 hours of collection to prevent fungal overgrowth or degradation during storage. Refrigeration was continuously maintained until the samples were processed to preserve their integrity. The geographic coordinates of the sampling locations were recorded to ensure the reproducibility of the study.

Media Preparation

To isolate fungi, Potato Dextrose Agar (PDA) was used as the growth medium. A mixture of 39g of PDA powder was dissolved in 1000 ml of distilled water in a conical flask. The solution was autoclaved at 121°C for 15 minutes under 15 lbs of pressure. After autoclaving, the medium was allowed to cool slightly, enriched with 250mg of chloramphenicol capsule to inhibit bacterial growth, and then dispensed into sterile Petri dishes. All procedures were carried out in a sterile environment to prevent contamination.

Isolation of Fungi

Samples collected from the phyllosphere and rhizosphere were incubated on PDA plates for 5 days at 25°C. The leaf samples were washed in 10% sodium hypochlorite, followed by rinsing in distilled water. The cleaned samples were then placed on PDA media, centred on the plate. Soil samples underwent serial dilution: a 1g soil sample was diluted in 9 ml of distilled water to create the stock solution. Dilutions (10^{-1} to 10^{-4}) were made, and 1 ml from the 10^{-4} dilution was transferred onto PDA plates. The inoculated plates were incubated for 5 days at 25°C. All procedures, including dilution and inoculation, were performed in replicate to ensure reliable results.

Purification and Preservation of Fungi

After incubation, visible fungal colonies were transferred to fresh PDA plates for purification. Colonies were isolated by sub-culturing until pure cultures were obtained. These pure cultures were stored at 4°C in a refrigerated environment for long-term preservation.

Identification of Fungi Species

Fungal identification was performed using standard microscopic techniques. A portion of the fungal hyphae from the pure cultures was placed on a sterile glass slide and stained with Lactophenol cotton blue. A cover slip was placed over the slide, and the excess stain was gently removed with blotting paper. The sample was then observed under a microscope, and identification was based on the morphological and cultural characteristics of the fungal species. Identification was confirmed with reference to fungal taxonomic keys and databases.

Pathogenicity Test

The pathogenicity of the isolated fungi was assessed by preparing spore suspensions under sterile conditions. A 5g sample of the fungal isolate was mixed with 45 ml of sterile distilled water, mechanically agitated, and filtered to remove large particulate matter. The spore concentration, determined using a hemocytometer, was then applied to cassava plants grown in autoclaved loamy soil from the University of Lagos botanical garden. Non-inoculated plants, treated identically but receiving sterile distilled water in place of the spore suspension, served as negative controls. A

separate pathogenicity assessment was conducted by applying the spore suspension to the leaves of the plants. The inoculated plants, along with those containing test leaves, were enclosed in polythene bags for 2 days to maintain high humidity, after which they were left uncovered for an additional 5 days. Following the incubation period, symptoms such as chlorosis, necrotic lesions, and weakened stems were observed. Infected plant samples were subsequently collected and cultured on PDA plates to confirm fungal growth and complete the pathogenicity cycle, and establish Koch's Postulates (Adeogun *et al.*, 2025b; Matche and Adeogun, 2022; Siviero *et al.*, 2019; State, 2010).

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of *Mentha piperita* Oil

The volatile components of *Mentha piperita* essential oil were analysed using a PerkinElmer GC-MS instrument. Helium was used as the carrier gas with a split ratio of 1:200. Mass spectra were acquired over the range 35-500 m/z, and TurboMass 6.1.0 was used to process the spectra. Components were identified by comparing mass spectra to the NIST library, and their relative abundances were determined by integrating peak areas. This analysis allows for a comprehensive understanding of the chemical composition of peppermint oil (Adeogun *et al.*, 2025a; Adeogun *et al.*, 2018b, 2018a).

Preparation of Gelatin and Peppermint Formulation

The gelatin solution was prepared according to the method adopted by Adekunle *et al.* (2021), Adeogun *et al.* (2023), and Adeogun *et al.* (2025a) by dissolving 40g of gelatin in 305 mL of distilled water, mixed with 0.5 mL of glacial acetic acid, which was added to facilitate the dissolution of the gelatin. After ensuring complete dissolution, the solution was divided into four parts for the following test ratios: the 100% gelatin treatment (containing acetic acid at the same concentration used in all formulations) served as a vehicle control, allowing any antifungal activity observed in peppermint oil treatments to be attributed to the essential oil rather than to the gelatin matrix or acetic acid carrier.

- 100% gelatin (100 ml of gelatin)

- 98% gelatin + 2% peppermint essential oil (98 ml of gelatin + 2 ml peppermint oil)
- 96% gelatin + 4% peppermint essential oil (96 ml of gelatin + 4 ml peppermint oil)
- 90% gelatin + 10% peppermint essential oil (90 ml of gelatin + 10 ml peppermint oil)

Each ratio was thoroughly mixed in separate beakers to ensure a homogeneous solution.

***In Vitro* Antifungal Effects of Gelatin Incorporated with Peppermint Oil**

To assess the antifungal effects of different peppermint oil-to-gelatin ratios, as described by Adeogun *et al.* (2025a); Adeogun *et al.* (2023); Adeogun *et al.* (2025b), and Matche and Adeogun (2022), 17 ml of prepared PDA was combined with 3 ml of each test mixture (gelatin and peppermint oil), and the mixture was stirred until homogeneous. The resulting mixture was poured into sterile Petri dishes. Pure fungal cultures were then inoculated onto both control and test plates (each colony cultured on four test plates and one control plate). Petri dishes were incubated at 25°C for 10 days, with daily monitoring for fungal growth. Growth was measured and recorded to evaluate the antifungal activity of the different test mixtures.

Statistical Analysis

Data were analysed using two-way analysis of variance (ANOVA) to evaluate the main effects of the two independent factors and their interaction. When significant differences were detected, Tukey's honestly significant difference (HSD) post hoc test was applied for multiple pairwise comparisons at a significance level of $p \leq 0.05$. Results are expressed as means \pm standard deviation of four replicates. Graphs were plotted using GraphPad Prism (Version 8.4.3), and statistical analysis was performed using SPSS software (Version 26).

RESULTS

Fungi Diversity on *Manihot esculenta* (Cassava Plant)


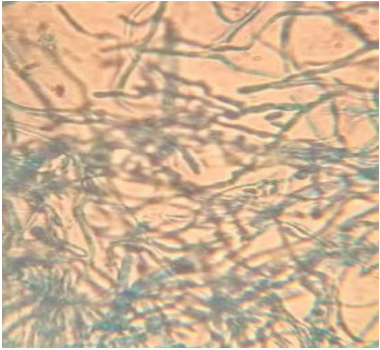

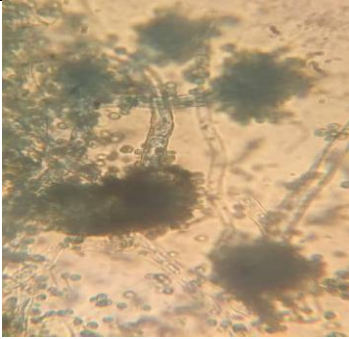
A total of six fungal species were isolated from *Manihot esculenta* (cassava) plants across the rhizoplane, rhizosphere, phylloplane, and phyllosphere habitats. All isolates were identified based on macroscopic colony


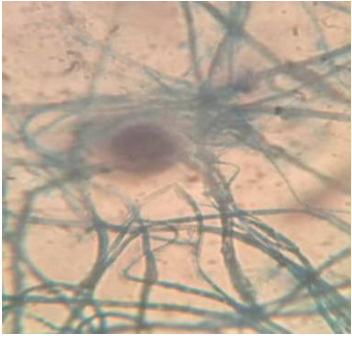

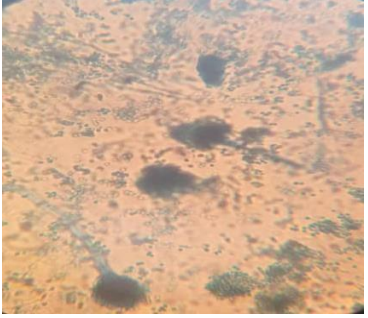

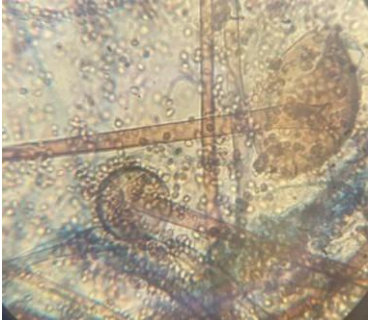
characteristics and microscopic examination at $\times 400$ magnification (Table 1).

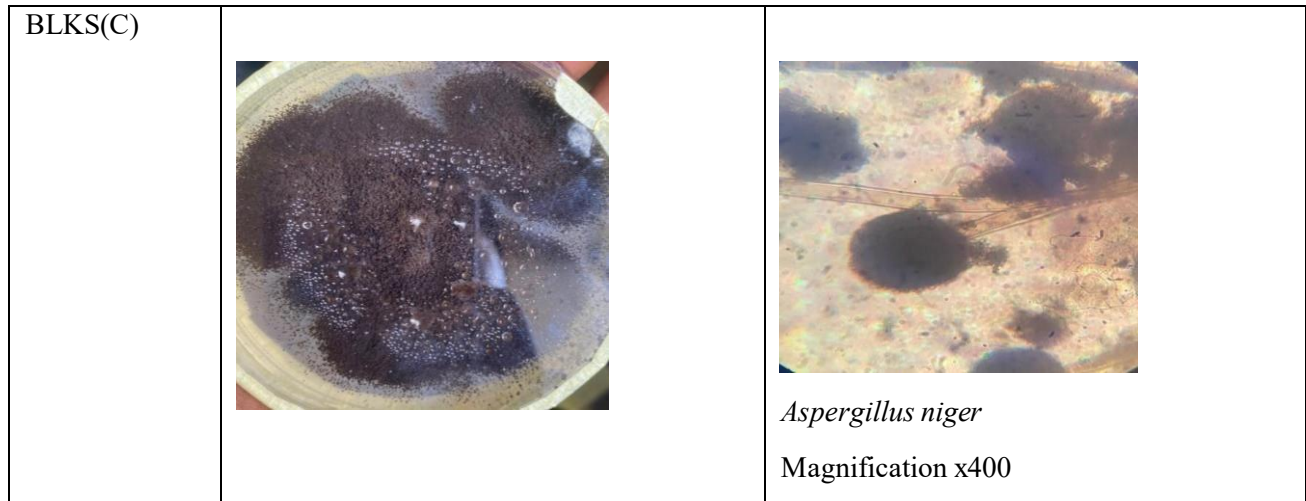
The isolate WHTL(E) was identified as *Colletotrichum* sp., based on its distinctive colonial morphology, with microscopic observation revealing features consistent with the genus. Similarly, GRL(B) was identified as *Aspergillus flavus*, as its cultural and microscopic characteristics aligned with established descriptions of the species. The isolate GYL(H) was identified as *Macrophomina phaseolina*, with microscopy confirming its characteristic structural features.

BL/GRS(G) was identified as *Penicillium* sp., recognised by its pigmentation and the typical brush-like arrangement of conidia observed under the microscope. *Rhizopus stolonifer* was recovered from isolate COTS(D) and identified based on its characteristic sporangial morphology. Finally, BLKS(C) was identified as *Aspergillus niger*, distinguished by its dark colony pigmentation and characteristic conidiophore structure under microscopic examination.

Table 1: Macroscopic and microscopic description of fungi isolated *Manihot esculenta*

NAME	FRONT VIEW	MICROSCOPIC VIEW (Magnification)
WHTL(E)		 <i>Colletotrichum</i> sp. Magnification x400
GRL(B)		 <i>Aspergillus flavus</i> Magnification x400

<p>GYL(H)</p>		 <p><i>Macrohomina phaseolina</i> Magnification x400</p>
<p>BL/GRS(G)</p>		 <p><i>Penicillium sp.</i> Magnification x400</p>
<p>COTS(D)</p>		 <p><i>Rhizopus stolonifer</i> Magnification x400</p>



Frequency of fungal colonies across the five months (November – March)

The frequency of fungal occurrence, as presented in Figures 1-4, indicated that the leaf surface mycobiota varied distinctly across the five sampling locations during the five-month study period (November–March). Fungal occurrence frequency is expressed as the number of months in which a species was isolated out of five total sampling months (e.g., 5/5 = isolated in all five months; 0/5 = not detected). *Aspergillus niger* and *Aspergillus flavus* were the most frequently isolated species.

Specifically, *A. flavus* exhibited the highest frequency (5/5 months) at the Stadium location and a high frequency (4/5 months) at Agura, whereas it was least frequent at Unilag (1/5 months). *Aspergillus niger* showed the greatest persistence at Agura (5/5 months) and the Stadium (4/5 months).

Colletotrichum sp. was moderately distributed, with the highest occurrence recorded at Unilag and Agura (4/5 months each). *Macrophomina phaseolina* exhibited the lowest frequency among the phylloplane isolates, peaking at 3/5 months at Unilag and the Stadium.

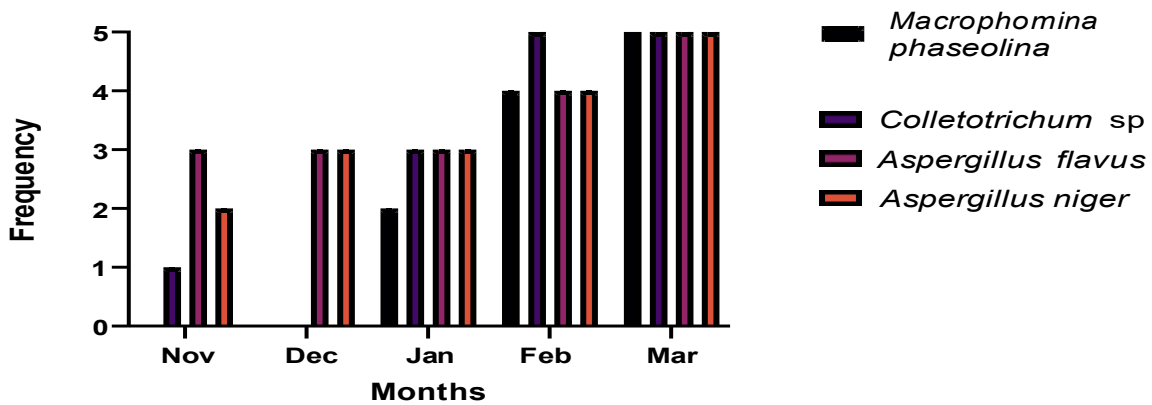


Fig. 1: Frequency of Fungal Colonies on the Phylloplane

In the phyllosphere, the mycoflora composition shifted, with the absence of *Colletotrichum* sp. and *Macrophomina phaseolina* and the emergence of *Rhizopus stolonifer*. *Rhizopus stolonifer* was highly dominant, appearing in all

five sampling months (5/5) at Agura, Imota, and the Stadium.

Aspergillus niger was consistently present across all locations, ranging from 3/5 to 4/5 months. Notably, *Aspergillus flavus* showed significant spatial variation; while it was frequently isolated

at Agura and the Stadium (4/5), it was completely undetectable (0/5) in the

phyllosphere at Unilag throughout the study period.

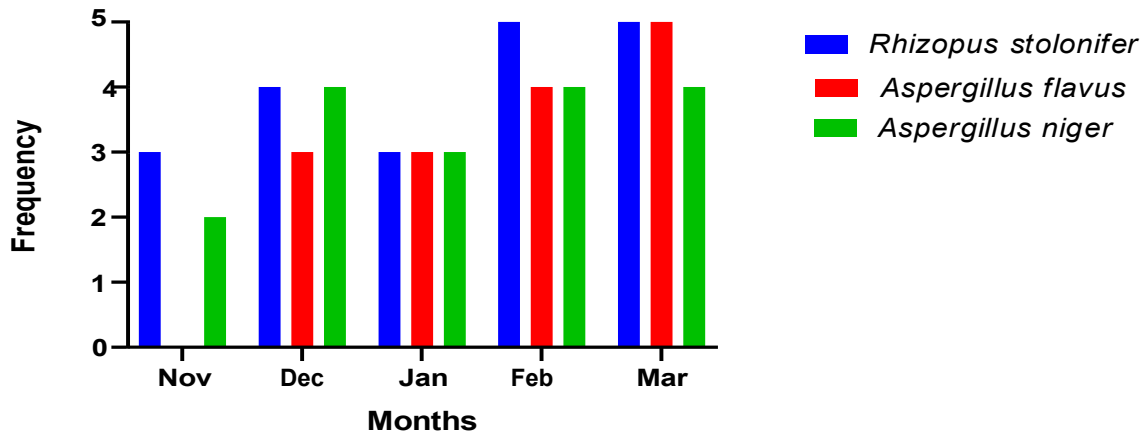


Fig. 2: Frequency of Fungal Colonies within the Phyllosphere

The rhizoplane was characterized by the ubiquitous presence of *Rhizopus stolonifer*, which was isolated in 100% of the sampling months (5/5) at Agura, Imota, and Unilag, and 4/5 months at the Stadium. This zone also showed the presence of *Penicillium* sp., which was observed at low-to-moderate frequencies (2/5 to 3/5) across all locations.

Aspergillus niger remained a persistent colonizer on root surfaces, particularly at Unilag, Imota, and Agura (4/5). *Aspergillus flavus* was most frequent at Ijede and the Stadium (4/5), with moderate occurrence (2/5 to 3/5) observed at other sites.

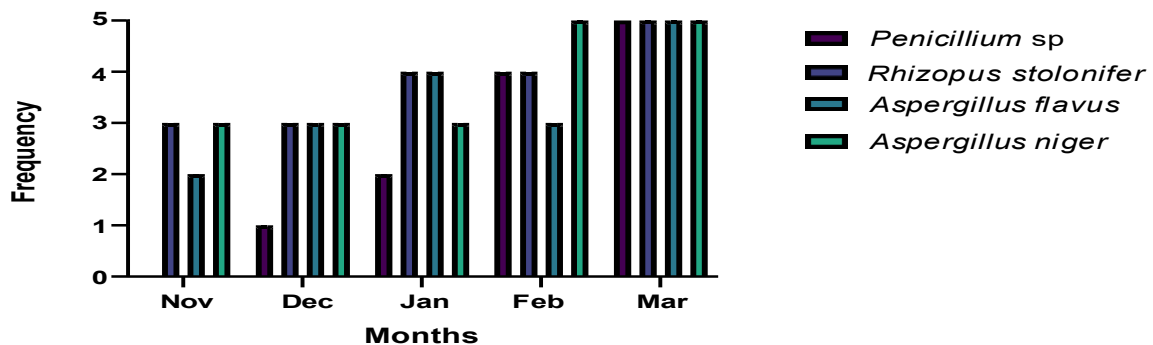


Fig. 3: Frequency of Fungal Colonies on the Rhizoplane

The rhizosphere mycoflora showed patterns similar to those of the rhizoplane, but with variations in intensity. *Rhizopus stolonifer* remained the dominant isolate, recording a 5/5 frequency at Agura and Unilag, though it dropped to 2/5 at Ijede.

Aspergillus niger was most prevalent in the soil at Imota (5/5) and the Stadium (4/5), while occurring at a moderate frequency (3/5) across Agura, Ijede, and Unilag. *Aspergillus flavus* displayed a relatively uniform distribution across most locations (3/5), with a slightly lower incidence at the Stadium (2/5). Overall, Ijede

demonstrated the lowest aggregate frequency of fungal colonies in the rhizosphere, with no single species exceeding a 3/5 occurrence rate. Species diversity was quantified for each habitat using Shannon’s diversity index (H') and Simpson’s index (D), calculated from the occurrence frequency data. The computed values were: Phylloplane ($H' = 1.3493$, $D = 0.7314$), Phyllosphere ($H' = 1.0916$, $D = 0.6620$), Rhizoplane ($H' = 1.3708$, $D = 0.7427$), and

Rhizosphere ($H' = 1.0872$, $D = 0.6593$). These indices confirm that the Rhizoplane harboured the greatest species evenness and diversity, while the Rhizosphere exhibited the least diversity, dominated by fewer fungal species. The Phylloplane also showed relatively high diversity, whereas the Phyllosphere was intermediate but less even than the Phylloplane and Rhizoplane.

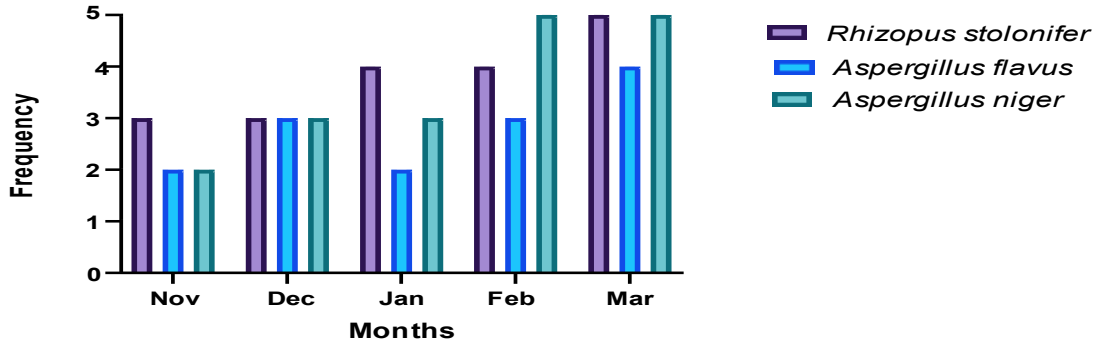


Fig. 4: Frequency of Fungal Colonies on the Rhizosphere

Pathogenicity of Fungal Isolates on *Manihot esculenta*

The pathogenicity test, as illustrated in Figures 5a-e, confirmed the virulence of the fungal isolates obtained from *Manihot esculenta*. *Colletotrichum* sp. and *Macrophomina phaseolina*, which had previously been isolated from *M. esculenta* during the initial isolation process, were re-isolated after inoculation and confirmed to be the same organisms. Initial

signs of infection were observed two days after inoculation. Disease progression increased markedly over the next 48 hours, and the inoculated plants exhibited typical symptoms of fungal infection, including severe leaf wilting, chlorosis, and foliar desiccation. The development of these symptoms in the inoculated *M. esculenta* plants demonstrated the pathogenicity of the *Colletotrichum* sp. and *Macrophomina phaseolina* isolates.



Fig 5a: Application of Spore



Fig. 5b: Opening of inoculated plants after 2 days



Fig 5c: Inoculated cassava plant exposed after 2 days



Figs. 5d and 5e Inoculated plants with fungal symptoms

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis of *Mentha piperita* Oil

Table 2 shows that *Mentha piperita* oil contains 15 main constituents. The GC-MS analysis revealed that γ -Terpineol (39.83%) was the most

abundant component, followed by 1-Menthone (27.35%) and Cyclohexanone, 5-methyl-2-(methylethyl) (9.68%). The constituents of *Mentha piperita* (peppermint) essential oil were analyzed and are tabulated as shown below:

TABLE 2: GC-MS constituents of *Mentha piperita* Oil

S/N	RETENTION TIME	CONSTITUENTS	RETENTION INDEX	(%) OCCURRENCE
1.	6.090	(-)-trans-pinane	847	0.148
2.	6.470	α -Pinene	933	1.520
3.	7.550	(+)-Sabinene	899	0.314
4.	8.991	o-Cymene	887	0.109
5.	9.126	D-Limonene	919	5.380
6.	9.191	Eucalyptol	916	0.170
7.	10.391	1,2 -Oxolinalool	857	0.221
8.	10.857	Ethyl 2-(5-methyl-5-vinyl tetrahydrofuran-2-yl) propan-2-yl-carbonate	849	0.147
9.	11.192	β -linalool	883	0.157
10.	12.872	1-Menthone	951	27.35
11.	13.137	Cyclohexanone, 5-methyl-2-(methylethyl)	883	9.684
12.	13.272	Menthol	847	0.138
13.	13.503	γ -Terpineol	967	39.83
14.	13.883	δ -Terpineol	880	1.599
15.	14.033	4,7 -dimethylundecane	848	2.087

In vitro Assessment of Peppermint Oil and Gelatin on Fungi isolated from Phylloplane, Phyllosphere, Rhizoplane and Rhizosphere Habitats

Fig. 6 shows the effect of pure PDA (control), PDA + 100% gelatin, and gelatin combined with varying concentrations of peppermint oil on the mycelial growth of *Aspergillus flavus* during a 10-day incubation period. The vertical bar represents the mean of four replicates. The control group (PDA alone) exhibited a continuous increase in mycelial growth from 0.45 ± 0.06 cm on day 0 to 4.15 ± 0.03 cm on day 10, while the positive control (PDA + 100% gelatin) also demonstrated a steady increase from 0.37 ± 0.05 cm on day 0 to 4.05 ± 0.03 cm

on day 10. In contrast, treatments containing various concentrations of gelatin and peppermint oil (PDA + GEL98% + PEO2%, PDA + GEL96% + PEO4%, PDA + GEL90% + PEO10%) showed minimal and consistent growth throughout the 10-day observation period. Treatment concentration, time, and their interaction all had statistically significant effects on mycelial growth (two-way ANOVA; treatment effect: $F(4, 90) = 781.2$, $p < 0.0001$; time effect: $F(5, 90) = 178.6$, $p < 0.0001$; interaction: $F(20, 90) = 69.58$, $p < 0.0001$; Tukey's HSD post hoc test). Percentage inhibition at GEL90% + PEO10% on day 10 was 84.3%.

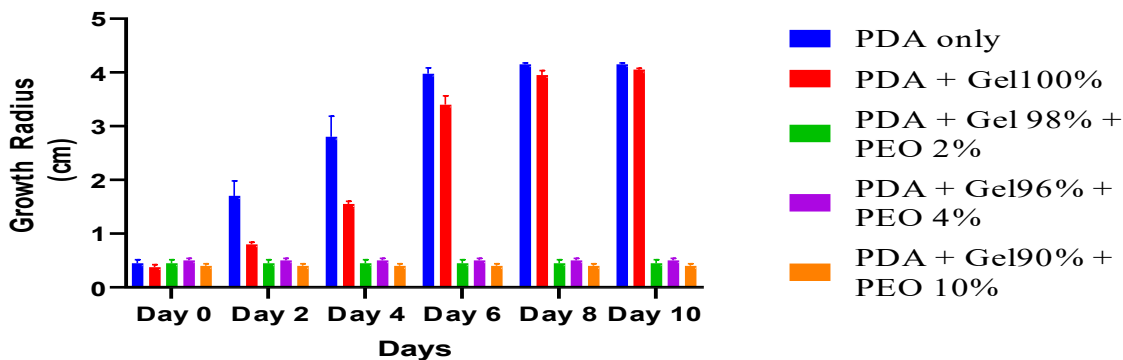


Fig. 6: The Effect of Gelatin integrated with varied concentrations of Peppermint Oil on the Mycelial Growth of *Aspergillus flavus*

Figure 7 shows the effect of pure PDA (control), PDA + 100% gelatin, and gelatin combined with varying ratios of peppermint oil on the mycelial growth of *Aspergillus niger* during a 10-day incubation period. The control group (PDA alone) exhibited a consistent increase in mycelial growth from 0.35 ± 0.06 cm on day 0 to 4.05 ± 0.06 cm on day 10, while the positive control (PDA + 100% gelatin) showed a steady increase from 0.40 ± 0.04 cm on day 0 to 2.63 ± 0.11 cm on day 10. In contrast, treatments containing varying concentrations of gelatin and peppermint oil (PDA + GEL98% + PEO2%,

PDA + GEL96% + PEO4%, PDA + GEL90% + PEO10%) displayed minimal and consistent growth throughout the 10-day observation period. Treatment concentration, time, and their interaction all had statistically significant effects on mycelial growth (two-way ANOVA; treatment effect: $F(4, 90) = 396.3$, $p < 0.0001$; time effect: $F(5, 90) = 54.25$, $p < 0.0001$; interaction: $F(20, 90) = 24.59$, $p < 0.0001$; Tukey's HSD post hoc test). Percentage inhibition at GEL90% + PEO10% on day 10 was 84.3% relative to the untreated control.

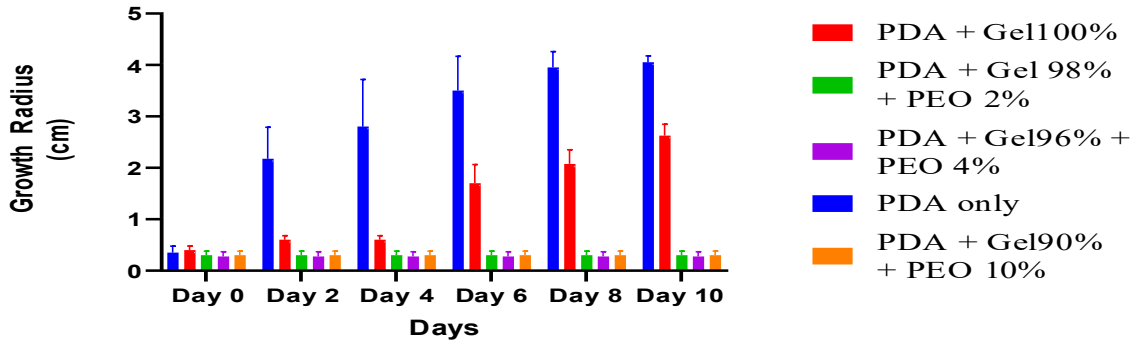


Fig. 7: The Effect of Gelatin integrated with varied concentrations of Peppermint Oil on the Mycelial Growth of *Aspergillus niger*

Figure 8 shows the effect of pure PDA (control), PDA + 100% gelatin, and gelatin combined with varying ratios of peppermint oil on the mycelial growth of *Rhizopus stolonifer* during a 10-day incubation period. The vertical bar represents the mean of four replicates. The control group (PDA alone) exhibited a steady increase in mycelial growth from 0.28 ± 0.05 cm on day 0 to 4.16 ± 0.03 cm on day 10, while the positive control (PDA + 100% gelatin) also showed a progressive increase from 0.45 ± 0.06 cm on day 0 to 4.08 ± 0.06 cm on day 10. In contrast, treatments containing different concentrations of

gelatin and peppermint oil (PDA + GEL98% + PEO2%, PDA + GEL96% + PEO4%, PDA + GEL90% + PEO10%) exhibited minimal and relatively constant growth throughout the 10-day observation period. Treatment concentration, time, and their interaction all had statistically significant effects on mycelial growth (two-way ANOVA; treatment effect: $F(4, 90) = 1483, p < 0.0001$; time effect: $F(5, 90) = 342.2, p < 0.0001$; interaction: $F(20, 90) = 131.2, p < 0.0001$; Tukey’s HSD post hoc test). Inhibition at GEL90% + PEO10% was 93.3% relative to the untreated control on day 10.

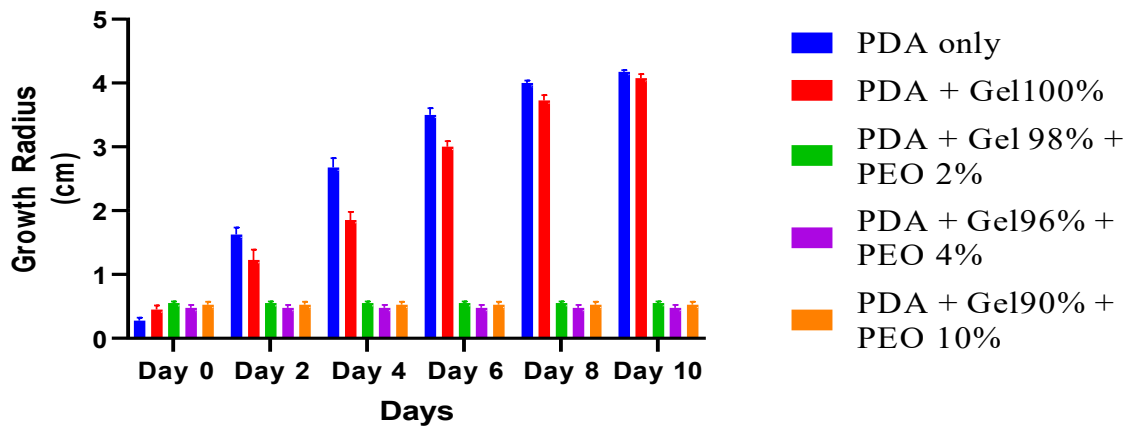


Fig. 8: The Effect of Gelatin integrated with varied concentrations of Peppermint Oil on the Mycelial Growth of *Rhizopus stolonifer*

Figure 9 shows the effect of pure PDA (control), PDA + 100% gelatin, and gelatin combined with varying ratios of peppermint oil on the mycelial growth of *Colletotrichum* sp. during a 10-day incubation period. The vertical bar represents the mean for four replicates. The control group

(PDA alone) exhibited a steady increase in mycelial growth from 0.45 ± 0.06 cm on day 0 to 4.15 ± 0.06 cm on day 10, while the positive control (PDA + 100% gelatin) showed a continuous increase from 0.35 ± 0.06 cm on day 0 to 4.10 ± 0.09 cm on day 10. In contrast, the

treatment samples containing different concentrations of gelatin and peppermint oil (PDA + GEL98% + PEO2%, PDA + GEL96% + PEO4%, PDA + GEL90% + PEO10%) exhibited minimal and consistent growth throughout the 10-day observation period. Treatment concentration, time, and their interaction all had statistically significant effects

on mycelial growth (two-way ANOVA; treatment effect: $F(4, 90) = 1323, p < 0.0001$; time effect: $F(5, 90) = 295.9, p < 0.0001$; interaction: $F(20, 90) = 120.0, p < 0.0001$; Tukey's HSD post hoc test). Percentage inhibition at GEL90% + PEO10% on day 10 was 91.6%.

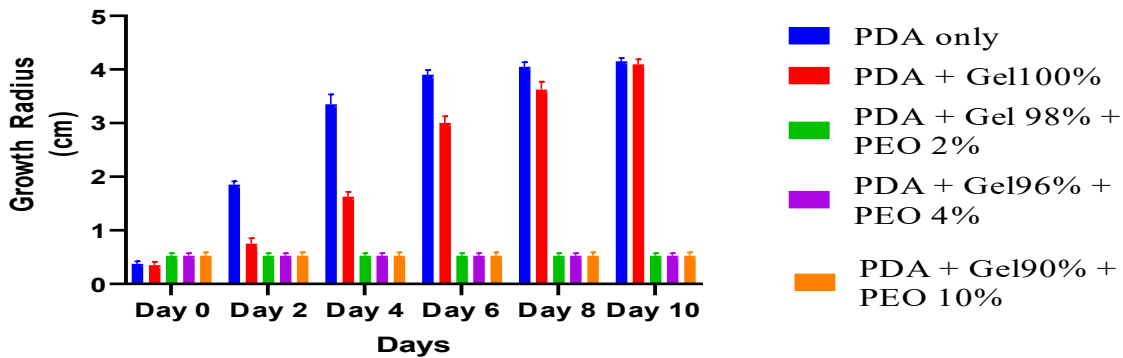


Fig. 9: The Effect of Gelatin integrated with varied concentrations of Peppermint Oil on the Mycelial Growth of *Colletotrichum sp.*

Figure 10 shows the effect of pure PDA (control), PDA + 100% gelatin, and gelatin combined with varying ratios of peppermint oil on the mycelial growth of *Penicillium sp* during a 10-day incubation period. The vertical bar represents the mean for four replicates. The control group (PDA alone) exhibited a steady increase in mycelial growth from 0.45 ± 0.06 cm on day 0 to 4.13 ± 0.06 cm on day 10, while the positive control (PDA + 100% gelatin) showed a continuous increase from 0.43 ± 0.04 cm on day 0 to 4.18 ± 0.09 cm on day 10. In contrast, the treatments containing gelatin-peppermint oil

formulations displayed minimal and consistent growth throughout the 10-day observation period. Treatment concentration, time, and their interaction all had statistically significant effects on mycelial growth (two-way ANOVA; treatment effect: $F(4, 90) = 2342, p < 0.0001$; time effect: $F(5, 90) = 548.9, p < 0.0001$; interaction: $F(20, 90) = 215.9, p < 0.0001$; Tukey's HSD post hoc test). Percentage inhibition at GEL90% + PEO10% on day 10 was 92.3%, confirming statistically significant inhibitory activity against *Penicillium sp.*

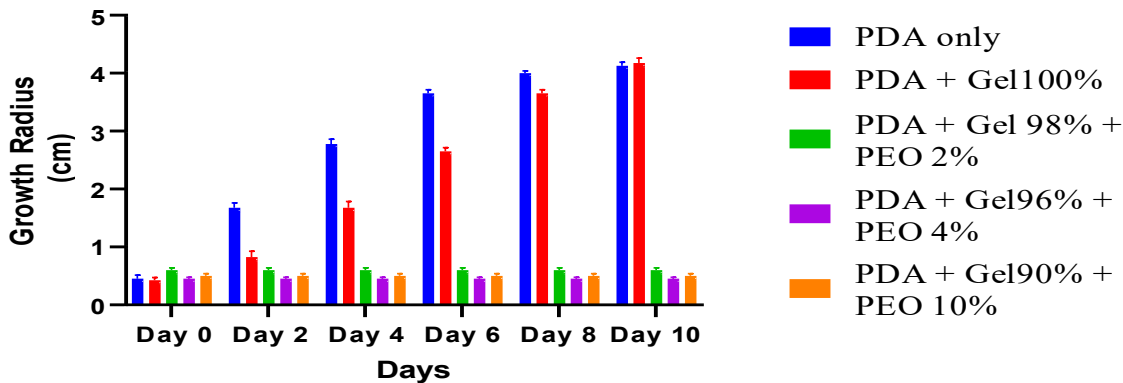


Fig. 10: The Effect of Gelatin integrated with varied concentrations of Peppermint Oil on the Mycelial Growth of *Penicillium sp*

Figure 11 shows the effect of pure PDA (control), PDA + 100% gelatin, and gelatin combined with varying ratios of peppermint oil on the mycelial growth of *Macrophomina phaseolina* during a 10-day incubation period. The vertical bar represents the mean radial growth for four replicates. The control group (PDA alone) exhibited a steady increase in mycelial growth from 0.40 ± 0.04 cm on day 0 to 4.00 ± 0.07 cm on day 10, while the positive control (PDA + 100% gelatin) showed a continuous increase from 0.45 ± 0.06 cm on day

0 to 4.03 ± 0.04 cm on day 10. In contrast, all other treatments demonstrated minimal and consistent growth throughout the 10-day observation period. Treatment concentration, time, and their interaction all had statistically significant effects on mycelial growth (two-way ANOVA; treatment effect: $F(4, 90) = 1515$, $p < 0.0001$; time effect: $F(5, 90) = 318.6$, $p < 0.0001$; interaction: $F(20, 90) = 120.1$, $p < 0.0001$; Tukey's HSD post hoc test). Percentage inhibition at GEL90% + PEO10% on day 10 was 92.5%.

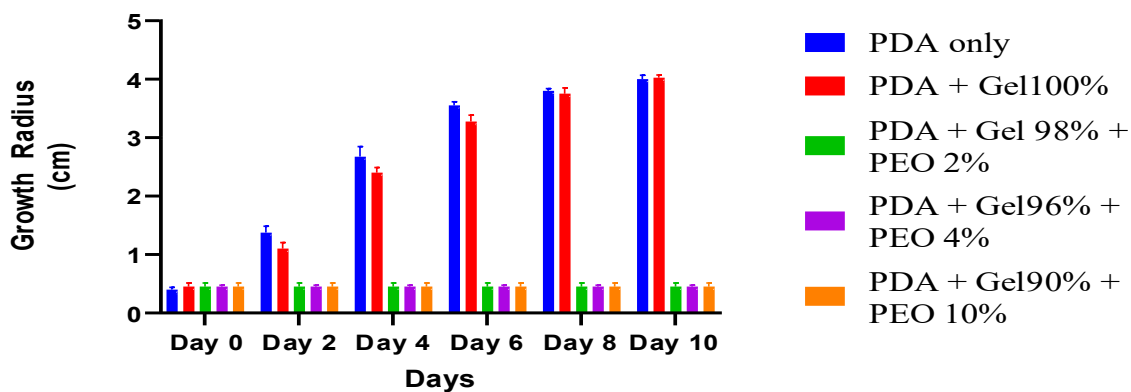


Fig. 11: The Effect of Gelatin integrated with varied concentrations of Peppermint Oil on the Mycelial Growth of *Macrophomina phaseolina*

DISCUSSION

The primary goal of this study was to evaluate the potential of gelatin coatings, both with and without peppermint oil, to inhibit the growth of pathogenic fungi on *Manihot esculenta* (cassava). While no prior research has explored the combined effects of gelatin and peppermint oil on fungal pathogens in cassava, our findings suggest that this combination holds promise as a natural solution for controlling fungal growth under in vitro conditions.

The fungal diversity observed on cassava, spanning the phylloplane, phyllosphere, rhizoplane, and rhizosphere, is closely tied to various plant diseases. Common pathogens, as posited by Marquez *et al.* (2021) and Olunoiki and Ayansina (2021), such as *Aspergillus niger* and *Aspergillus flavus*, can cause severe post-harvest spoilage, while *Rhizopus stolonifer* and *Macrophomina phaseolina* contribute to root rot and other issues, leading to yield loss.

Additionally, fungi such as *Colletotrichum* species cause anthracnose, which affects cassava leaves, stems, and fruits, sometimes resulting in significant yield losses. Under conducive conditions, these fungi can severely impact cassava growth, underscoring the need for effective control methods (Ferreira *et al.*, 2026; Machado *et al.*, 2024).

Conventional control strategies often rely on chemical fungicides, but these solutions raise concerns about environmental pollution, the development of resistance, and food safety. Therefore, exploring natural, sustainable alternatives is essential (Fenta and Mekonnen, 2024; McLaughlin *et al.*, 2023). Gelatin, being biodegradable and non-toxic, presents an excellent carrier for bioactive compounds. When combined with peppermint oil, which contains active antifungal constituents such as menthol, menthone, and limonene, it could offer a powerful, environmentally friendly alternative to

synthetic fungicides. Setiawan *et al.* (2025) confirm that peppermint leaves contain active constituents such as menthol, menthone, and limonene, known for their antifungal potency, and identify menthol and limonene as major constituents with antifungal properties.

Our study showed that gelatin combined with peppermint oil had significant antifungal activity against the fungal species tested, including *Aspergillus flavus*, *Penicillium* sp., *Colletotrichum* sp., *Macrophomina phaseolina*, *Aspergillus niger*, and *Rhizopus stolonifer*. The peppermint oil's antifungal compounds likely contribute to this activity by disrupting fungal cell membranes and inhibiting their growth—a mechanism proposed based on the terpenoid composition of peppermint oil but not directly validated in this study. These results align with previous studies, such as Prakash *et al.* (2012), which demonstrated the antifungal efficacy of essential oils, including peppermint oil, against *Aspergillus flavus*. In our study, the control groups (untreated and gelatin-only) showed steady fungal growth, while treatments with gelatin and peppermint oil consistently exhibited minimal growth, reinforcing the inhibitory effects of this formulation.

Similarly, our findings align with those of Freire *et al.* (2012) and Phillips *et al.* (2012), who reported that citrus and peppermint essential oils effectively inhibit the growth of *Aspergillus niger*. In both studies, the essential oils disrupted fungal growth, albeit from different plant sources: peppermint and citrus, suggesting that essential oils share common mechanisms of fungal inhibition. These results suggest that peppermint oil, when combined with gelatin, can provide a robust, sustainable solution for managing *A. niger* in post-harvest settings and can be further tested through field validation.

The antifungal efficacy of peppermint oil against *Rhizopus stolonifer* observed in our study is supported by existing literature. Several studies have demonstrated the potent inhibitory effects of *Mentha* species essential oils against this pathogen. Vakili-Ghartavol *et al.* (2022) reported significant mycelial growth suppression with *Mentha piperita* oil at varying concentrations, which aligns with findings by Uzsáková *et al.* (2022) and Tančinová *et al.* (2018), who documented complete inhibition of

multiple *Rhizopus* isolates, positioning peppermint oil among the most effective essential oils tested.

The use of gelatin as an encapsulation matrix in our study addresses key challenges with direct essential oil application, particularly volatility and stability. Adeogun and Jonas (2026) offer a comparable framework, demonstrating that gelatin-encapsulated peppermint oil at 1%, 3%, and 5% concentrations achieved significant to complete fungal growth inhibition. Their research showed that gelatin matrices enhance oil stability and enable sustained antifungal release—factors likely contributing to the effectiveness of our tested concentrations of 2%, 4%, and 10%. The antifungal activity is primarily attributed to menthol and menthone, the major constituents of peppermint oil (Edris and Farrag, 2003).

Our results with the peppermint oil-gelatin formulation against *Colletotrichum* and *Penicillium* species align with existing research. Peralta-Ruiz *et al.* (2020) demonstrated strong antifungal effects using essential oils combined with chitosan against *Colletotrichum gloeosporioides*, mirroring our findings with *Colletotrichum* species on cassava. Both studies confirm that essential oils, when paired with natural matrices, effectively inhibit fungal growth.

Several studies support peppermint oil's efficacy against *Colletotrichum* species. Andrade and Vieira (2016) achieved complete inhibition of *C. gloeosporioides* mycelial growth and spore germination on papaya using 100 μ L peppermint oil, while Ramos *et al.* (2016) reported fungicidal activity at a 12.5% concentration. Dong *et al.* (2019) found higher concentrations were required against *C. acutatum* (minimum inhibitory concentration of 20 μ L/mL), but the evidence consistently supports moderate to strong antifungal activity against *Colletotrichum*.

Regarding *Penicillium* species, our results showed robust growth suppression, in contrast to Moritz *et al.* (2020), who reported only partial effects of clove essential oil. This reinforces peppermint oil's strong anti-*Penicillium* activity. Vakili-Ghartavol *et al.* (2022) demonstrated dose-dependent inhibition of *P. expansum* at 250–2000 ppm, with severe morphological

damage. Their subsequent work (2024) showed that encapsulated peppermint oil achieved 89% inhibition, compared to 67% for free oil. Valková *et al.* (2021) and Mrvová *et al.* (2024) confirmed complete inhibition of multiple *Penicillium* species.

The enhanced effectiveness observed in our study is likely due to the gelatin delivery system. Adeogun and Jonas (2026) demonstrated that gelatin-encapsulated peppermint oil at 1%, 3%, and 5% significantly inhibited phylloplane fungi, with complete suppression at the higher concentrations. Importantly, the gelatin matrix improved oil stability and provided sustained antifungal release. Additional studies by Scartazzini *et al.* (2019) and Tian *et al.* (2025) further validated the efficacy of gelatin-based delivery systems, with the latter achieving a 100% reduction in *Penicillium* on cherry tomatoes using a 2.5% peppermint oil emulsion. The peppermint oil-gelatin formulation also proved effective against *Macrophomina phaseolina*, the causative agent of charcoal rot. This result aligns with established research on peppermint oil's antifungal activity against *M. phaseolina*. El-shoraky and Shala (2018) reported 89.6% mycelial inhibition, with a significant reduction in sclerotial formation, while Khaledi *et al.* (2015) demonstrated 66.7–100% disease reduction under greenhouse conditions, affecting both fungal growth and cell wall-degrading enzymes. Additional studies have shown consistent dose-dependent inhibitory effects, with peppermint oil outperforming other essential oils against this pathogen (Ghazi *et al.*, 2018; Moghaddam *et al.*, 2013).

Our formulation notably maintained inhibitory effects over 10 days, surpassing the 5-day activity reported for lavender oil by Marchidan *et al.* (2023). This extended effectiveness is likely due to the controlled-release properties of the gelatin matrix, as demonstrated by Adeogun and Jonas (2026), who found that gelatin encapsulation enhanced peppermint oil stability and provided sustained release. Recent encapsulation studies by Mokhtari *et al.* (2025) and Liu *et al.* (2025) further support this, showing sustained antifungal activity over 120 days. While peppermint oil is typically considered fungistatic rather than fungicidal

against *M. phaseolina*, our gelatin-based delivery system appears to overcome this limitation by providing prolonged exposure and maintaining effective concentrations over extended periods.

CONCLUSION

This study demonstrates the *in vitro* antifungal activity of a gelatin and peppermint oil formulation against six fungal species associated with *Manihot esculenta* across four habitat zones. The notable inhibitory efficacy observed, particularly against *Aspergillus* species and *Rhizopus stolonifer*, with GEL90% + PEO10% achieving 84.3% and 93.3% growth suppression respectively, highlights its potential as a sustainable alternative to synthetic fungicides in cassava postharvest management. The controlled-release properties of gelatin enhanced the stability and prolonged the antifungal activity of peppermint oil over the 10 day *in vitro* observation period. As fungal resistance to synthetic fungicides continues to rise, these findings support the investigation of gelatin and peppermint oil formulations as components of integrated disease management strategies for cassava and other crops. Field trials are required before recommendations for large-scale practical application can be made. While this study focused on six dominant fungal species recovered through culture-based isolation, future investigations incorporating metagenomic approaches may reveal additional members of the cassava mycobiome that were not culturable under the conditions employed, further broadening the ecological context of the findings reported here.

ETHICAL CLEARANCE

No experiments involving humans or animals were conducted in this study. Therefore, ethical approval was not required.

CONFLICT OF INTEREST

We, the authors, declare that there are no conflicts of interest, either financial or non-financial, related to this study. We have no affiliations, funding sources, or personal interests that could be perceived as influencing the research or its findings.

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