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Antifungal Potential of Chitosan and Phenyllactic Acid against *Colletotrichum gloeosporioides*, the Anthracnose Pathogen in Avocados

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Abstract

Avocado (*Persea americana* Mill.) is a tropical fruit with significant economic value worldwide. However, the avocado industry faces important challenges from anthracnose rot, caused by *Colletotrichum gloeosporioides* species, which affects negatively fruit quality and market value. Growing concerns about synthetic fungicides and consumer demand for safer alternatives drive interest in natural bioactive agents for postharvest disease control. This research assessed the *in vitro* antifungal efficacy of chitosan (CHI), phenyllactic acid (PLA), individually and in combination, against *C. gloeosporioides* isolated from Kenyan avocados. Through the poisoned food assay, both CHI and PLA significantly ($P < 0.05$) inhibited pathogen growth in a concentration-dependent manner. PLA completely (100%) inhibited mycelial growth and conidia germination at 4 mg/mL, while CHI achieved 78.82% inhibition of mycelial growth and 66.67% inhibition of conidia germination at 15 mg/mL. CHI-PLA mixtures exhibited an additive type of interaction (Abbott method) and caused significant morphological deformation of conidia. These findings highlight CHI-PLA mixtures as a promising and sustainable alternative to synthetic fungicides for managing postharvest anthracnose in avocados.

Keywords: Chitosan; Phenyllactic-acid; Anthracnose; *Colletotrichum gloeosporioides*; Avocado; Biocontrol

Introduction

Avocado (*Persea americana* Mill.) is an economically valuable fruit widely cultivated in the tropical and subtropical regions. It is recommended as a dietary source of mono-unsaturated fatty acids, fibers, vitamins, carbohydrates, and other essential nutrients. It has been referred to as a heart-healthy fruit.

In Kenya, avocado is the 4th most important fruit after banana, mango, and pineapple, contributing significantly to the country's GDP (HCDA 2020). However, postharvest losses primarily arise from anthracnose disease, mainly caused by *Colletotrichum gloeosporioides,* remain a significant challenge to the avocado industry. This disease appears as black spots on the avocado fruits' pericarp, severely affecting their quality and marketability (Kimaru *et al*. 2018b).

Traditionally, synthetic fungicides have been employed to manage anthracnose disease. However, their application is considered non-sustainable due to their negative impact on the environment and consumer health (Kimaru *et al*. 2020; Mpeluza *et al*. 2023). As a result, there is growing interest in environmentally friendly alternatives, such as cold storage, modified atmosphere packaging (Xing *et al*. 2016), plant extracts (Chege and Kimaru 2021), essential oils (Bill *et al*. 2017) and edible coatings (Bautista-Baños *et al*. 2003). Among these, edible coatings have gained considerable attention owing to their potential in enhancing fruit quality and shelf life while being safe and eco-friendly (Bautista-Baños *et al*. 2003; Oliveira *et al.* 2017).

Chitosan (CHI), a biodegradable polymer derived from chitin deacetylation, is known for its antimicrobial effects and potential as an edible coating (Li *et al*. 2024). Research demonstrates that CHI concentrations of 1.5% and above can effectively control fungal growth (Bautista-Baños *et al*. 2003; Marques *et al*. 2016). However, higher concentrations may adversely impact fruit ripening by restricting gas exchange and causing physiological disorders (Ali *et al*. 2010). As a result, combining CHI with other

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bioactive compounds, such as plant extracts, organic acids, and essential oils, has been explored to enhance its antifungal and coating properties (Li *et al*. 2024).

Phenyllactic acid (PLA) is an organic acid and natural metabolite of Lactic Acid Bacteria known for its antimicrobial activity and non-cytotoxicity (Rajanikar *et al*. 2021). Although PLA has not yet been approved as a food additive, its inhibitory efficacy against various foodborne pathogens and its safety profile make it a promising candidate for food preservation. Recent studies have demonstrated PLA's potential in reducing anthracnose in bananas, suggesting that its combination with a coating material like CHI could offer synergistic protection against similar fungal infections (Gao *et al*. 2024).

Therefore, this study investigated the antifungal potential of CHI, PLA, and their combination against *C. gloeosporioides* isolated from avocado fruits, with the aim of developing a sustainable approach for managing postharvest anthracnose. the Combination of PLA and CHI is hypothesized to improve antifungal activity, as PLA's ability to penetrate fungal cells (Rajanikar *et al.* 2021) may complement CHI's action in disrupting fungal cell membranes (Oliveira *et al.* 2017).

Materials and Methods

Experimental materials

CHI (biological reagent, degree of deacetylation ≥ 90%, Mw= 100,000 Da; Cat. No. C850348) and PLA (Cat. No. YS122518) were obtained from Solarbio Sciences and Technology Co., Ltd. (Beijing, China). Lactic acid was obtained from Thomas Scientific Company (Logan Township, US). Avocado fruits (*Hass* cv.) were collected at commercial maturity from a commercial orchard in Gatundu, Kiambu, Kenya.

Isolation and identification of *Colletotrichum gloeosporioides* isolates

Isolation

Colletotrichum gloeosporioides isolates were obtained from naturally infected mature avocado fruits collected from avocado orchards in Gatundu, Kiambu, Kenya (0°55′44.60″S 36°52′25.64″E). The isolation process was conducted based on the methods described by Chowdappa *et al*. (2012) and Kimaru *et al*. (2018a).

Morphological and microscopic identification

C. gloeosporioides isolates were morphologically characterized following the published descriptions by Barnett and Hunter (1972), Chowdappa *et al*. (2012) and Weir *et al*. (2012). The features assessed included cultural characteristics of the colonies on PDA and microscopic characteristics of conidia, focusing on their shape and size.

Pathogenicity test

A pathogenicity test was conducted using healthy, mature Hass avocado fruits sourced from an avocado orchard within the study area to confirm Koch's postulates (Liu *et al*. 2015). The fruits were surface-sterilized with 0.5% sodium hypochlorite and inoculated with mycelial plugs collected from actively growing cultures of the isolates on PDA medium. Control fruits were inoculated with PDA plugs only. Two fruits per isolate were used, with two replicate wounds on each fruit. The inoculated avocado fruits were placed on sterile plastic trays lined with moistened paper towels, covered with cling film to maintain high humidity level, and incubated at room temperature (22 ± 4°C) under normal light conditions for six days.

Molecular identification

The isolates that produced typical anthracnose symptoms from the pathogenicity test were identified at the molecular level by sequencing the Internal Transcribed Spacer (ITS) region of the rDNA using the universal primers ITS1-F (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4-R (5'-TCCTCCGCTTATTGATATGC-3') at Macrogen Ltd, Singapore. Fungal genomic DNA was extracted using the Zymo Quick-DNA™ Fungal/Bacterial Miniprep kit (Inqaba Biotech, South Africa) following to the manufacturer's protocol.

Conventional PCR amplification was carried out in a Biometra Trio thermocycler (Analytik Jena, Germany) under the following thermal cycling conditions:

- Initial denaturation at 94°C for 10 min
- 34 cycles of:
  - Denaturation at 94°C for 70 sec
  - Annealing at 56°C for 60 sec
  - Polymerization at 68°C for 60 sec
- Final extension at 68°C for 5 min

The resulting DNA sequences were assembled into consensus sequences using BioEdit software. These sequences were then subjected to nucleotide homology searches in the GenBank database using the BLAST program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the identity of the isolates. Subsequently, a phylogenetic tree was constructed using Mega11 software to determine the relationships with other *C. gloeosporioides* species complex retrieved from the Genbank database.

In vitro antifungal experiments

The *C gloeosporioides* isolate, which demonstrated high rapid growth and pathogenicity, was selected for the *in vitro* experiments.

Preparation of CHI solution and PLA

The CHI solution was prepared following the methods described by Bautista-Banos *et al*. (2003) and Li *et al*. (2009).

To prepare a 10 mg/mL CHI solution, 1 g of CHI powder was dissolved in 100 mL sterile distilled water containing 1.5% lactic acid. Preliminary experiment indicated that lactic acid was optimal for CHI activity and did not significantly affect the growth of *C. gloeosporioides* isolates compared to other acids like glacial acetic acid and formic acid, as also confirmed by Li *et al*. (2009). The solution was stirred continuously on a magnetic stirrer at room temperature overnight to ensure complete dissolution, and the pH was adjusted to 5.6 using 2 N NaOH. The tested concentrations of CHI were 5, 10 and 15 mg/mL. To achieve the desired concentrations of CHI in the PDA medium, a double-strength CHI solution was prepared and diluted with an equal volume of molten PDA.

The direct incorporation method was adopted for the antifungal tests with PLA alone. PLA powder was weighed and incorporated directly into molten PDA to obtain final concentrations of 1, 2, 3 and 4 mg/mL.

For the combined application, the CHI solution was prepared as described above. However, for the PLA, a portion of the sterile distilled water (20 mL) intended for dissolving the PDA medium was used to dissolve the required amount of PLA to achieve the desired final concentration in the mixture. This PLA solution was then incorporated into the CHI solution and mixed for at least 30 min under constant stirring until a stable, homogeneous solution was obtained.

Inhibitory activity of CHI and PLA on the mycelia growth rate of *C. gloeosporioides*

The inhibitory potential of CHI and PLA, both individually and in combination, on radial mycelial growth rate of *C. gloeosporioides* isolates was examined through the poisoned food technique as previously described by Li *et al*. (2009), Oliveira *et al*. (2017) and Silva et *al*. (2021).

A 6 mm mycelial plug excised from the edge of a 4-day-old culture of *C. gloeosporioides* on PDA was used to inoculate the center of Petri plates containing PDA amended with CHI, PLA, or a CHI-PLA combination at the desired final concentrations. The inoculated plates were then placed in the incubator at 28 ± 2°C. As positive control, the PDA medium was amended with the fungicide Milraz 70 WP (a mixture of Propineb 700 g/kg and Cymoxanil 60 g/kg) at the manufacturer's recommended concentration (2 mg/mL). The negative control consisted of PDA without any treatment. Additionally, a PDA medium supplemented with an aqueous solution of 1.5% lactic acid (pH 5.6) was considered as a negative control for chitosan tests. The experiments consisted of three experimental replicates for each treatment and were repeated two times.

The colony diameters were measured along two perpendicular axes every two days until the negative control plates were filled with mycelia (six days after inoculation). The Mycelial Growth Inhibition percent (MGI%) was computed using the formula:

$$MGI\% = \frac{Mc-Mt}{Mc} \times 100$$

Mc is the Mycelia diameter in control plates while Mt is the Mycelia diameter in treatment plates.

Inhibitory activity of CHI and PLA on conidia germination

This assay was conducted following the methods of Meng *et al*. (2010) and Chowdappa *et al*. (2012). This experiment aimed to determine whether the treatment could inhibit the germination of *C. gloeosporioides* conidia, which could have infected the avocado fruits in the orchard as part of a strategy for postharvest control of anthracnose.

A pre-experiment (data not shown) on avocado fruits identified CHI (15 mg/mL), PLA (4 mg/mL), and their combination as the optimal concentrations for postharvest application. Therefore, these concentrations were selected for the conidia germination inhibition assays.

A conidial suspension (1 x 10^6 conidia/mL), adjusted using an Improved Neubauer Haemocytometer, was prepared from 7-day-old *C. gloeosporioides* cultures. 35 µL of the suspension was inoculated onto a glass slide containing solidified PDA medium (200 μL) supplemented with either CHI (15 mg/mL), PLA (4 mg/mL), or a combination of CHI-PLA (15 mg/mL + 4 mg/mL) and evenly spread using a sterile hockey stick spreader. Milraz, applied at the manufacturer's recommended concentration of 2 mg/mL, served as the positive control, and untreated PDA as the negative control. The inoculated slides were put in sterile Petri plates aligned with a moistened paper tissue and then incubated at 28 ± 2°C for 12 h. Solid media were chosen for this assay, as conidia failed to germinate in pre-experiments conducted in either Potato Dextrose Broth (PDB) medium or sterile distilled water.

Germination inhibition was assessed by counting 100 conidia per replicate, using three experimental replicates for each treatment, under a light microscope at X40 magnification. A conidium is deemed germinated if its germ tube was at least as long as the conidium itself. The Germination inhibition percentage (GI%) due to the treatments was computed using the formula:

$$GI\% = \frac{Gc-Gt}{Gc} \times 100$$

Gc is the number of conidia that germinated in the control group, and Gt is the number of conidia that germinated in the treated group.

Type of interaction between CHI and PLA

The type of interaction between CHI and PLA when combined was evaluated through the Abbott method (Oliveira *et al*. 2017; Li *et al*. 2024). The following formulas were used:

$$MGI\%exp = MGI_{CHI}\%obs + MGI_{PLA}\%obs - \left(\frac{MGI_{CHI}\%obs \times MGI_{PLA}\%obs}{100}\right)$$

MGI_{PLA}%obs and MGI_{CHI}%obs are the observed mycelial growth inhibition percentages when CHI and PLA were applied individually at a given concentration.

The Abbott Index (**AI**) was then determined by:

$$AI = \frac{MGI\%obs}{MGI\%exp}$$

MGI%obs is the observed mycelial growth inhibition when CHI-PLA were applied in combination.

Interpretation of AI values was as follows: a synergistic effect if $AI \geq 1.5$, an additive effect if $0.5 \leq AI < 1.5$ and an antagonistic effect if $AI < 0.5$.

Statistical analysis

Data were analyzed using descriptive statistics, analysis of variance (ANOVA) in Minitab software, and graphs were generated using OriginPro software. Data were presented as the mean of three experimental replicates ± Standard Error of the Mean (SEM) and Tukey's test was applied at 95% confidence level to identify significant differences.

Results

Identification and pathogenicity of *C. gloeosporioides* isolates

All obtained isolates exhibited cottony mycelia with white to light-grey coloration on the upper surface of PDA, which darkened to grey with age. The mycelia displayed a raised and sparse hyphal structure, growing in a circular pattern with a regular, white margin. They also displayed an orange conidial mass near the inoculation point and a creamy-white color at the culture's underside (Fig. 1). Under the microscope, these isolates produced hyaline, one-celled, cylindrical conidia with either both ends rounded or one end round and the other pointed (Table 1; Fig. 2). These observed morphological characteristics are consistent with those previously reported for the *C. gloeosporioides* species (Barnett and Hunter 1972; Chowdappa *et al.* 2012; Weir *et al*. 2012).

Pathogenicity tests on mature *Hass cv.* avocado fruits inoculated with *C. gloeosporioides* isolates resulted in the development of brown to black, sunken, water-soaked necrotic lesions that spread radially (Fig. 3). This typical anthracnose symptom was produced by all tested isolates, with variations in lesion diameter (Table 2). Koch's postulates were confirmed by re-isolating the pathogen from symptomatic avocado fruits and re-identifying them based on their morphological characteristics.

PCR amplification with ITS1 and ITS4 primers produced amplicons of approximately 560 bp. The partial DNA sequences obtained after Sanger sequencing showed a 100% homology with *C. gloeosporioides* species DNA sequences present in the GenBank. These sequences were later deposited in the GenBank database under the accession numbers PQ317978 to PQ317983. Phylogenetic analysis using the Neighbor-Joining method further demonstrated 100% relatedness to reference sequences of *C. gloeosporioides* retrieved from GenBank (Fig. 4). Moreover, the sequences also displayed a strong genetic relationship with *C. fructicola, C. aenigma* and *C. siamense* species, which belong to the *C. gloeosporioides* species (Weir *et al.* 2012).

Table 1: Mycelia growth rate and conidia size of the isolates on PDA

Isolates	Daily Mean MGR (mm/Day)	Conidia Size (μm)	
		Length	Width
CG1-k	7.72 ± 0.06^{b}	15.39 ± 0.41^{bc}	4.20 ± 0.16^{c}
CG2-K	7.72 ± 0.06^{b}	16.19 ± 0.30^{ab}	4.93 ± 0.11^{ab}
CG3-K	7.72 ± 0.06^{b}	16.91 ± 0.47^{a}	5.27 ± 0.19^{a}
CG4-K	8.22 ± 0.06^{a}	14.19 ± 0.21^{c}	4.07 ± 0.18^{c}
CG5-K	8.00 ± 0.17^{ab}	15.08 ± 0.29^{bc}	4.56 ± 0.11^{bc}
CG6-K	7.69 ± 0.03^{b}	17.66 ± 0.44^{a}	4.37 ± 0.08^{bc}

The values are expressed as mean ± SE of Mean. Means that share similar letter are not significantly different based on Tukey's test at 95% confidence level

Table 2: Disease incidence and lesion diameter of anthracnose from the pathogenicity test

Isolates	Disease Incidence (%)	Mean Lesion Diameter (mm)
CG1-K	100	21.67 ± 1.01^{ab}
CG2-K	100	15.67 ± 0.93^{c}
CG3-K	100	22.67 ± 0.33^{a}
CG4-K	100	22.67 ± 1.36^{a}
CG5-K	100	17.00 ± 1.53^{bc}
CG6-K	100	14.50 ± 1.44^{c}

The values are expressed as mean ± SE of Mean. Means that share similar letters are not significantly different ($P > 0.05$) based on Tukey's test

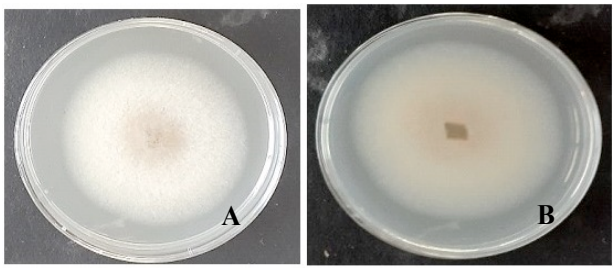

Fig. 1: 4-days-old pure culture of *C. gloeosporioides* on PDA. (**A)** Upper surface of the culture and (**B)** Reverse side of the culture

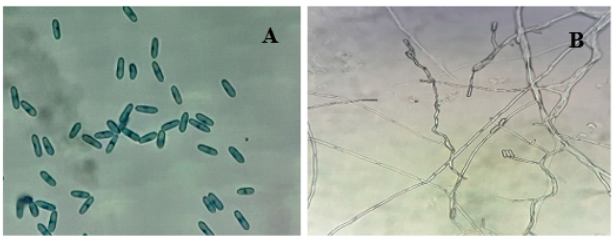

Fig. 2: Conidia of *C. gloeosporioides* under microscopy at 40x magnification. (**A**) Conidia stained with lactophenol cotton blue, (**B**) Unstained conidia borne on top of conidiophore

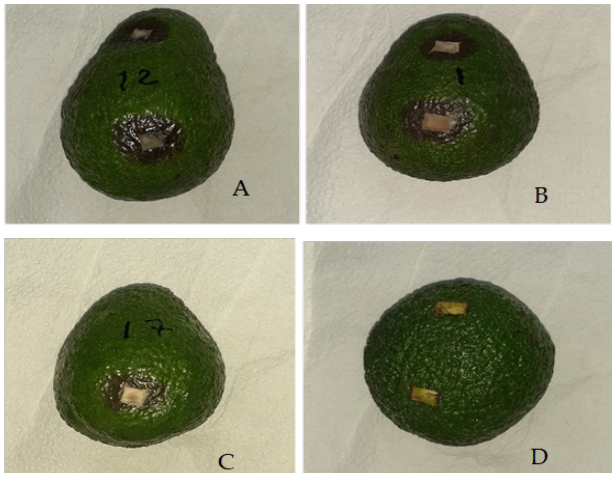

Fig. 3: Anthracnose symptoms produced by *C. gloeosporioides* on inoculated *Hass* avocado fruits 4 days post-inoculation. Infected fruit inoculated with isolate (**A**) CG4-k, (**B**) CG1-k, (**C**) CG5-k and (**D**) negative control

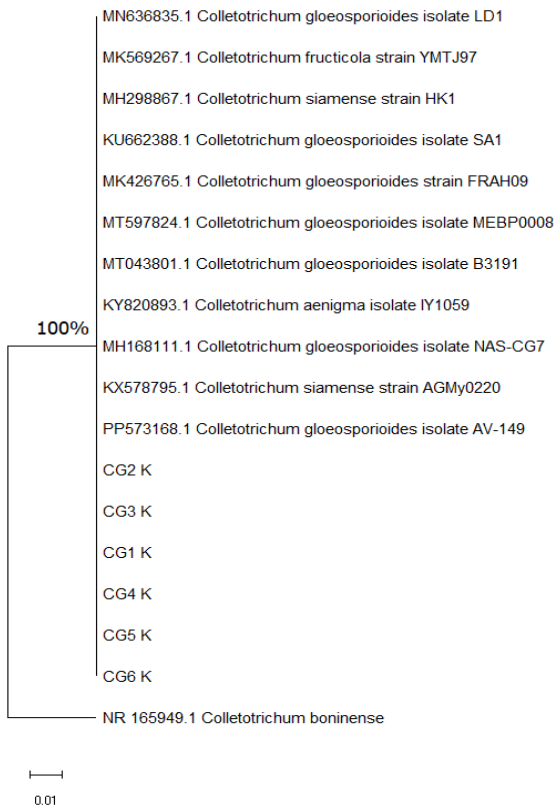

Fig. 4: Neighbor-joining phylogenetic tree showing the relationship of *C. gloeosporioides* isolates to related species based on ITS gene sequences. The scale bar 0.01 indicates substitutions per nucleotide position

Effect of CHI, PLA and CHI-PLA on radial mycelia growth rate of *C. gloeosporioides*

All tested concentrations of PLA and CHI demonstrated significant ($P < 0.05$) inhibitory potential on mycelial growth of *C. gloeosporioides* 6 days post-inoculation (Fig. 5). The inhibition percentage of mycelial growth rate increased in a dose-dependent manner, with the highest inhibition percentage obtained at the highest tested concentrations. A complete inhibition (100%) was achieved at the highest tested concentration of PLA (4 mg/mL). In contrast, CHI did not achieve complete inhibition even at the highest concentration of 15 mg/mL. At this concentration, CHI reduced the mycelial growth of *C. gloeosporioides* by 78.82% 6 days post-inoculation compared to the control. Furthermore, the MGI% achieved by the positive control, Milraz, was statistically ($P > 0.05$) comparable to that of PLA at 2 mg/mL and CHI at 10 mg/mL.

Using the Abbott method, the combination of PLA and CHI consistently exhibited an additive effect across all tested concentrations (Table 3). The antifungal activity of CHI decreased when PLA was added at low concentrations, but this activity improved as the PLA concentration increased. Despite this improvement, no complete mycelial growth inhibition was obtained in any of the combined treatments, even at the highest tested concentrations of both PLA and CHI. The Abbott Index (AI) for the combination treatments ranged from 0.5 to 0.9, indicating an additive interaction rather than a synergistic effect.

Effect of the treatments on conidia germination

All treatments exhibited inhibitory effects on conidia germination. PLA (4 mg/mL), the CHI-PLA (15 mg/ mL + 4 mg/mL) combination, and the positive control completely (100%) inhibited conidia germination 12 h post-inoculation, while CHI (15 mg/mL) alone inhibited germination by 66.67% (Fig. 6A). In addition, the CHI-PLA combination caused visible deformation (swelling) of the conidia (Fig. 6B1), a phenomenon not observed in the other treatments.

Discussion

Identification of *C. gloeosporioides*

C. gloeosporioides is among the most commonly reported pathogens associated with anthracnose disease in fruits and vegetables (Gautam 2014). In Kenya, for instance, Kimaru *et al*. (2018a) identified *C. gloeosporioides, C. boninense*, and *Pestalotiopsis microspora* as the anthracnose causal agents in avocado fruits in Murang'a county, Kenya. In contrast, this study identified only *C. gloeosporioides* as the anthracnose-causing agent in Kiambu County, Kenya. It is possible that, had a larger number of isolates been examined as in Kimaru *et al*. (2018a), the other two species might have also been detected. However, this study specifically aimed to investigate the antifungal potential of CHI, PLA, and CHI-PLA against *C. gloeosporioides* in avocado, which constrained the scope of pathogen identification.

Table 3: Effect of CHI-PLA combination on *C. gloeosporioides* mycelia growth rate and the type of effect

Treatments (mg/mL)	Mycelia Growth Inhibition (%)	Abbott Index	Type of Effect
CHI-5 + PLA-1	33.20 ± 0.45^{i}	0.5	Additive
CHI-5 + PLA-2	43.18 ± 0.12^{h}	0.57	Additive
CHI-5 + PLA-3	54.18 ± 0.36^{f}	0.58	Additive
CHI-5 + PLA-4	74.13 ± 0.44^{b}	0.74	Additive
CHI-10 + PLA-1	47.56 ± 1.22^{g}	0.65	Additive
CHI-10 + PLA-2	47.86 ± 0.77^{g}	0.59	Additive
CHI-10 + PLA-3	58.11 ± 0.18^{e}	0.62	Additive
CHI-10 + PLA-4	74.34 ± 0.67^{b}	0.75	Additive
CHI-15 + PLA-1	79.43 ± 0.78^{a}	0.91	Additive
CHI-15 + PLA-2	$68.03 \pm 1..02^{c}$	0.75	Additive
CHI-15 + PLA-3	62.12 ± 0.65^{d}	0.64	Additive
CHI-15 + PLA-4	73.12 ± 0.40^{b}	0.73	Additive

The data are expressed as mean ± SEM. Means that share similar letters do not significantly differ ($P > 0.05$) based on Tukey's test

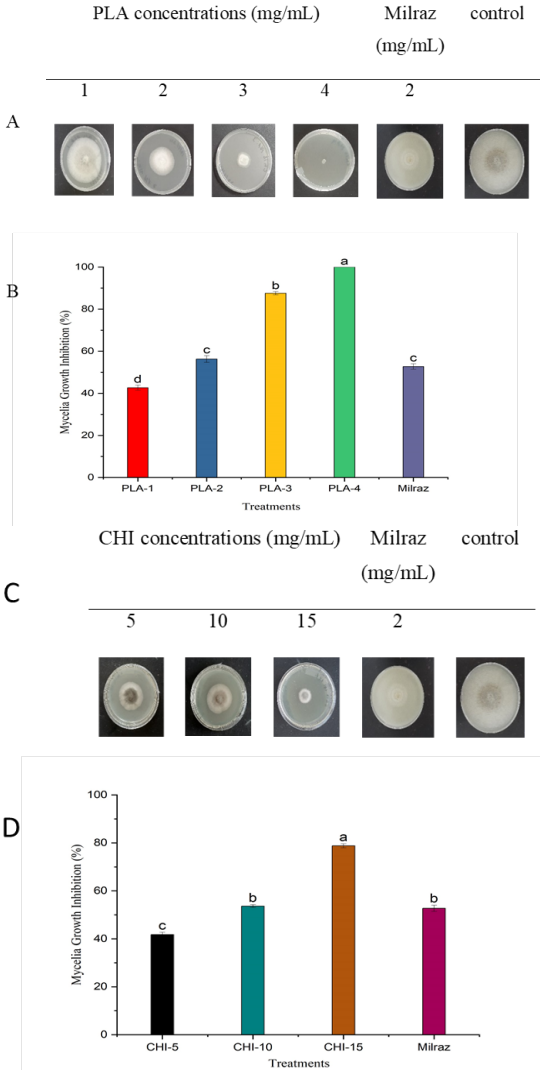

Fig. 5: Radial growth of *C. gloeosporioides* on PDA amended with phenyllactic acid (PLA) (**A**) & chitosan (CHI) (**C**), MGI% by PLA (**B**) and CHI (**D**). Error bars represent the SEM of three replicates. Means that share similar letters do not differ significantly ($P > 0.05$) based on Tukey's test

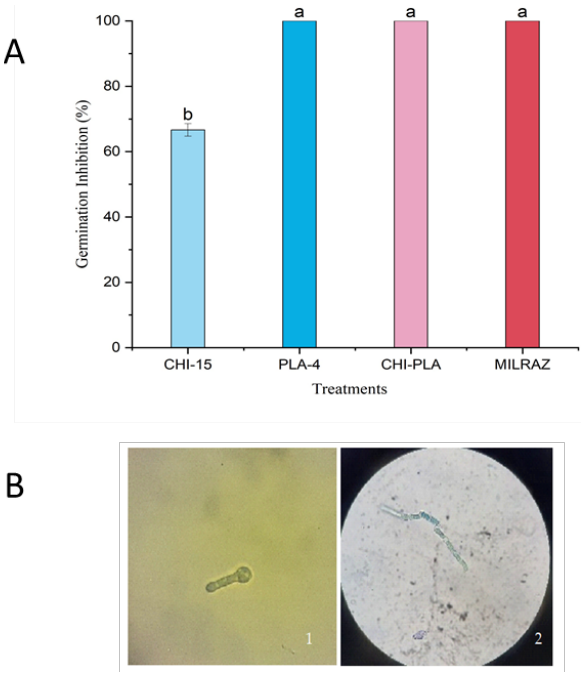

Fig. 6: Germination Inhibition % by phenyllactic acid (PLA at 4 mg/mL), chitosan (CHI at 15 mg/mL), CHI-PLA (15 mg/mL + 4 mg/mL) and Milraz (2 mg/mL) (**A**); Morphological deformation of conidium in CHI-PLA treatment (**B1**), **(B2)** typical germinated conidium. Error bars represent the SEM of three replicates. Means that share similar letters do not significantly differ ($P > 0.05$) based on Tukey's test

Future investigations should explore the diversity of fungal pathogens causing anthracnose disease in avocado fruits in Kiambu County.

In this study, *C. gloeosporioides* exhibited a close relationship with other *Colletotrichum* species such as *C. fructicola, C. aenigma, and C. siamense,* all of which are classified within the *C. gloeosporioides* species complex (Weir *et al*. 2012) due to their similar morphology and ITS sequences (Damm *et al*. 2010). Previous studies highlighted the limitation of relying solely on ITS sequence data for species identification and emphasized the need to use at least 2 to 3 genetic loci (such as GAPDH, ACT, TUB2) for accurate differentiation within the *C. gloeosporioides* species complex (Damm *et al.* 2010; Weir *et al.* 2012).

In vitro antifungal efficacy of CHI, PLA and CHI-PLA against *C. gloeosporioides*

Both CHI and PLA exhibited a concentration-dependent inhibitory activity against *C. gloeosporioides in vitro*, aligning with previous findings (Bautista-Baños *et al*. 2003; Lavermicocca *et al*. 2003; Oliveira *et al*. 2017; Guimarães and Venancio 2018; Gao *et al*. 2024). For instance, Bautista-Baños *et al*. (2003) reported complete inhibition of *C. gloeosporioides* mycelial growth at 25 mg/mL of CHI and significant conidia deformation at 15 mg/mL.

Similarly, Ali *et al*. (2010) observed 90% inhibition of radial mycelia growth and 80% inhibition of conidia germination at 15 mg/mL of CHI, with complete inhibition achieved at 20 mg/mL. The study also noted morphological changes in conidia following treatment with these CHI concentrations 7 h post incubation. Additionally, Oliveira *et al*. (2017) reported 100% inhibition of radial mycelia growth on other *Colletotrichum* species at 10 mg/mL of CHI.

PLA has also demonstrated strong antifungal potential. Gao et *al*. (2024) reported 96.56% inhibition of *C. musae* at 3 mg/mL. They found PLA effective in controlling anthracnose disease in bananas and suggested that its combination with a carrier material (like CHI) may improve its inhibitory potential. To date, however, no studies have evaluated the efficacy of the CHI-PLA combination against *C. gloeosporioides*. Therefore, this study is the first to investigate the efficacy of CHI and PLA combination against *C. gloeosporioides*. Similarly, Guimarães and Venancio (2018) reported complete inhibition of *Penicillium nordicum* at 8 mg/mL of PLA, while Cortés-Zavaleta *et al.* (2014) determined the minimum inhibitory concentration (MIC) of PLA against *C. gloeosporioides* to be 0.5 mg/mL. These studies highlight PLA's potential as a potent antifungal agent.

In the present study, PLA at 4 mg/mL achieved complete inhibition of both radial mycelia growth and conidia germination of *C. gloeosporioides in vitro*, whereas CHI did not reach full inhibition even at its highest tested concentration (15 mg/mL). The selected concentration of CHI is supported by previous findings suggesting its effectiveness as a postharvest fruit coating (Ali *et al*. 2010; Weir *et al*. 2012; Obianom *et al*. 2019). Moreover, the treatments' ability to inhibit conidial germination demonstrates their potential to target different stages of pathogen development. This capability is particularly significant, as inhibiting conidial germination could prevent disease progression in naturally infected fruits, enhancing postharvest disease management strategies.

CHI antifungal action is well-established and is mainly ascribed to its positively charged amino groups, which interact with the fungal plasma membrane's negatively charged phospholipids. This interaction disrupts membrane integrity, causing the leakage of intracellular components and cell death (Bautista-Baños *et al.* 2003; Oliveira *et al*. 2017). In contrast, PLA likely penetrates the cell membrane and dissociates intracellularly, releasing protons that acidify the cell and depolarize the membrane. This disrupts the cell's electrochemical balance, leading to key enzyme and metabolic pathways essential for cell survival inhibition and, ultimately, cell death (Guo *et al*. 2021; Ning *et al*. 2021; Rajanikar *et al*. 2021; Fan *et al.* 2022; Li *et al*. 2022; Zhang *et al*. 2024).

Recent iTRAQ-based proteomic studies have revealed that PLA also disrupts mitochondrial energy metabolism and glucose catabolism, leading to reduced energy supply, cytochrome C release, and apoptosis (Guo *et al*. 2021; Ning *et al*. 2021; Fan *et al.* 2022; Zhang *et al*. 2024). This dual action, disrupting membrane integrity and metabolic pathways, makes PLA a promising antifungal agent, especially when combined with other bioactive compounds like CHI.

In this study, CHI and PLA combinations exhibited consistent additive effects across all tested concentrations, as determined by the Abbott method. Interestingly, the antifungal efficacy of CHI was reduced when combined with low concentrations of PLA but increased as PLA concentrations were raised. Nevertheless, complete inhibition (100%) was not achieved in any of the combinations, unlike the effect observed with PLA at 4 mg/mL. This additive interaction observed under *in vitro* conditions indicates that while both agents are effective together, they do not significantly amplify each other's activities as would be expected in a synergistic interaction. A similar additive effect was reported by Li et *al*. (2024), where CHI and Iturin-A, when combined, exhibited an additive effect *in vitro* but demonstrated synergistic effects when applied to control *C. gloeosporioides* on mangoes.

Spectroscopic analyses (FTIR, HNMR and XRD) have shown that CHI and phenolic acid (such as gallic, vanillic, ferulic, salicylic acids) may interact through hydrogen bonds involving the hydroxyl or amine molecules of CHI and the carboxyl group of phenolic acid (Chatterjee *et al*. 2015; Wang *et al*. 2019; Liu *et al*. 2021). Similarly, through investigation of the antioxidant and antimicrobial (on some bacterial strains and *S. cerevisiae* and *P. expansum*) activity of PLA-grafted-CHI, Li *et al*. (2017) demonstrated that PLA and CHI could also interact through hydrogen bonds. The interaction of PLA with CHI may reduce the number of cationic amino groups available in CHI, potentially decreasing its antifungal efficacy. However, as PLA concentrations increase, the free PLA that does not interact with CHI functional groups may penetrate fungal cells and exert its antifungal activity, as seen in this study with higher PLA concentrations (Li *et al*. 2017).

Nevertheless, postharvest applications involve more complex biological environments, where interactions between the treatment compounds, fruit tissues, and pathogens may influence efficacy. These complexities often result in outcomes that differ from those observed under *in vitro* conditions. Notably, some studies have reported synergistic effects emerging under *in vivo* conditions despite additive responses *in vitro* (Li *et al*. 2024). Thus, future work will focus on optimizing the postharvest application of the CHI–PLA treatment on avocado fruits to assess their practical application.

Conclusion

This study demonstrated that CHI and PLA, both individually and in combination, significantly inhibited the mycelial growth and conidia germination of *C. gloeosporioides,* the anthracnose causal agent in avocados,

under *in vitro* conditions. PLA at 4 mg/mL completely inhibited both mycelial growth and conidia germination of *C. gloeosporioides*, while CHI (15 mg/mL) reduced mycelial growth by 78.82%, and conidia germination by 66.67%. The different mixtures of CHI and PLA exhibited an additive type of interaction based on the Abbott method. These results highlight the potential of CHI-PLA mixtures as a promising coating treatment to control anthracnose in avocados. Future research will focus on testing the CHI-PLA formulations on fresh avocado fruits to validate their practical application.

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Author's Contributions

HAH was responsible for conceptualizing, conducting the study, collecting data, analyzing the data, and writing the manuscript. WOO and SK contributed equally and were responsible for reviewing and validating the work, and as Supervisors.

Conflict of Interest

The authors declare no conflict of interest concerning this study.

Data Availability

Data will be made available upon reasonable request.

Ethics Approval

Not applicable to this paper.

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