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IN VITRO EFFICACY OF THREE POTENTIAL BIOAGENTS AGAINST *LASIODIPLODIA THEOBROMAE*, CAUSAL AGENT OF POSTHARVEST FRUIT DETERIORATION OF AVOCADO

Short communication

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ABSTRACT

The postharvest rot fungus, *Lasiodiplodia theobromae,* poses a significant threat to the avocado trade in Ghana as it discounts both the quality and shelf life of freshly harvested avocado fruits. This study evaluated the biological effect of three bioagents, garlic, baking soda, and black pepper, on mitigating postharvest rots of avocado fruits caused by *L. theobromae.* Isolates of *L. theobromae* obtained from spoiled fruits were identified using morphological, molecular, and pathogenicity procedures. Eight isolates of *L. theobromae* were similar in morphology and pathogenicity and genetic markers (ITS). The three bioagents, garlic and pepper seed powder, and baking soda, were evaluated *in vitro* using the food poison technique at two concentrations, 10 g and 20 g per 100 ml of water. Garlic at 20 g/100 ml of water completely inhibited mycelium growth, followed with 10 g/l (7.8%), and baking soda at 20 g/l (32.6%). Further studies of these bioagents on fruit are recommended.

Key words: biocontrol, garlic powder, pathogenicity, pepper seed powder, sodium bicarbonate, stem-end-rot

INTRODUCTION

Avocado (*Persea americana*) is a member of the Lauraceae family. It is a perennial tree cultivated for its savory and nourishing fruits (Kendír & Köroğlu 2018). Avocados have a higher potassium and protein content than bananas. Moreover, it is rich in nutritional fiber, folate, and vitamins C, K, and E (Yasir et al. 2010; Clark 2019). According to Abraham et al. (2018), some indigenous people in Ghana use the fruit and other parts of the tree for medicinal purposes.

The fruits are harvested while still firm and green, but they must be matured for complete and uniform ripening following harvest (Rodríguez-López et al. 2017).

In the supply chain, fungi are a crucial factor in the post-harvest losses of avocado fruits, and their occurrence can impact the quality and shelf life of fresh produce (Galsurker et al. 2018; Herrera-González et al. 2021). *Lasiodiplodia theobromae* is a significant post-harvest plant pathogen widespread throughout tropical and subtropical regions of the world and primarily causes rot (Faber et al. 2007). Its wide range of hosts is thought to consist of more than 500 plant species (Domsch et al. 2007; Alam et al. 2020), causing major losses to avocado, mango, cocoa, banana, and yam farmers (Rieger 2006). In a study by Wanjiku et al. (2020), *L. theobromae* was associated with stem end rots of avocado fruits in Kenya.

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Chemical fungicides used as preharvest treatments have effectiveness controlled these infections. However, the frightening toxicity and long-lasting effects of these chemical fungicides and the harm they cause to the environment, human health, and biodiversity, make them intolerable (Han et al. 2020). Another crucial factor is the potential for pathogens to become chemical-resistant (Ons et al. 2020; Yang et al. 2021). Using plant extracts and microbial antagonists presents an alternative to chemical fungicides in managing fruit postharvest diseases (Matrose et al. 2021; Yang et al. 2021).

Biological control agents are believed to have a reputation for being essentially harmless. It explains why their usage, as opposed to chemical pesticides in agriculture is rising (Carmona-Hernandez et al. 2019). For example, *Moringa oleifera* extract was successful in limiting of stem-end rot of mango fruit (Yeo et al. 2023). Using extracts of *Datura stramonium* and *Eucalyptus camaldulensis* has also been shown to reduce the mycelial growth of *L. theobromae* (Perumal et al. 2016).

This study, therefore, sought to establish the biological effects of three bioagents, garlic powder (*Allium sativum*), baking soda (sodium bicarbonate), and black pepper seed powder (*Piper nigrum*), on suppressing the growth of *L. theobromae,* the causal agent of avocado fruit rot. It will aid in developing a sustainable postharvest management strategy to improve fruit quality and shelf life.

MATERIALS AND METHODS

Isolation of avocado fruit rot pathogen

Rotten avocado fruits were sampled from four markets within Kumasi and its environs in January and April 2022. The samples were collected into sterile containers and brought to the Plant Pathology Laboratory, CSIR-Crops Research Institute, Kumasi, Ghana, for isolation and identification. They were kept in the refrigerator at 4 °C until required. For each sample, pieces of tissue from the periphery of the rotten mesocarp of the fruit were cut, surface sterilized for 5 minutes with a 5% solution of sodium hypochlorite, and rinsed three times with sterile distilled water. The pieces were allowed to air-dry in a laminar flow chamber for 1 hour. They were plated on Potato Dextrose Agar (PDA), amended with ampicillin (250 mg per 500 ml of PDA), and incubated at 28 °C for three days. Fungal hyphae emanating from the plated tissues were transferred onto fresh PDA. The isolates were subcultured several times until pure isolates were obtained.

Morphological identification of avocado fruit rot fungal isolates

The cultural plates of isolates were identified based on a comprehensive analysis of morphological features such as colony color, colony texture, mycelial growth pattern, conidia shape, and conidia color. To ensure the highest level of accuracy, 7-day-old cultures were used for the identification, with the exception of the conidia descriptions, which were based on 21-day-old cultures. The observed morphological descriptions were then meticulously cross-referenced with the descriptions as published in the identification manual of Mathur and Kongsdal (2003).

Molecular identification of avocado fruit rot fungal isolates

The genomic DNA of *L. theobromae* isolates was extracted using the Cetyltrimethylammonium bromide (CTAB) method. A 560-bp target sequence from the internal transcribed spacer (ITS) region was amplified with PCR using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The identity of the PCR products was checked using the Sanger dideoxy chain termination sequencing method (Du et al. 2005). A basic local alignment search tool (BLAST) algorithm was employed to identify the isolate by comparing it to the data available in the National Centre for Biotechnology Information (NCBI). The *L. theobromae* isolate was later deposited at GenBank, and an accession number was obtained. Phylogenetic analysis was performed by constructing a tree using the Maximum Likelihood method (i.e., RAxML algorithm). Bootstrap resampling was used to assess node support, with values > 70% considered robust.

Pathogenicity of fungal isolates

The identified isolates were assessed for their ability to infect and cause rot on healthy avocado fruits of the susceptible Western Indian cultivar harvested at the near-ripening stage in April and May 2022. The fruits were surface sterilized with a 5% sodium hypochlorite solution and rinsed thoroughly under running water. They were allowed to air-dry in a laminar flow chamber for 1 hour. The clean avocado fruits were mechanically wounded by singly piercing the middle portion of the fruit with a sterile inoculation needle. For each isolate, the hyphal tip of a 10-day-old culture, taken with a sterile inoculating needle, was used to inoculate the wounded point of the fruit's exocarp. As a check, a wounded avocado fruit was inoculated with *Fusarium oxysporum*, isolated from a rice seed. Another check was the non-inoculation of a wounded avocado fruit. The inoculated fruits were incubated in a sterile, transparent plastic container with water-soaked cotton wool placed in it to provide a humid environment. Each treatment replication had five fruits placed in the same container. This setup was repeated three times and arranged in a completely randomized design. The fruits were assessed for the incidence of rot five days after inoculation. The rotten area of fruit (RAF) was measured for each treatment replication using ImageJ computer software (ImageJ 2020). The inoculated fungi were re-isolated to complete Koch's postulate, and their characteristics were compared to the previous descriptions.

In a separate setup, a randomly selected *L. theobromae* isolate was used to inoculate the stem end of the fruit. This was done to specifically assess the modes of entry of the pathogen into the avocado fruit, a crucial aspect in understanding the infection and rotting process .

In vitro* assay of bioagents against *L. theobromae

The bio-activities of garlic (*Allium sativum*), black pepper (*Piper nigrum*), and baking soda (sodium bicarbonate) were evaluated against *L. theobromae* isolate, PP373854. Garlic bulbs and black pepper seeds were oven-dried for three days at 75 °C. Following drying, the garlic bulbs and black pepper seeds were separately milled to obtain fine powders. Ten grams (10 g) and 20 g of each of the three bioagent powders were poured into 250-ml conical flasks. 100 ml of sterile distilled water was added and mixed thoroughly. One milliliter of the suspension was then pipetted onto the surface of a solidified PDA, smeared with a sterile glass rod, and allowed to dry for 30 minutes. The pathogen was then seeded onto the amended PDA centrally and incubated at 28 °C. Mycelial growth of the pathogen was measured every 24 hours for 4 days. Plates not surface-coated with any bioagent solution served as controls. Each treatment was replicated three times. Values of the mycelial growth after 96 h were used to calculate the Mycelial Growth Rate (MGR) for each treatment plot by dividing the mycelial growth of the fungus by the incubation period (i.e., 96 h). This experiment was done in May 2022 and repeated in June 2023 to confirm the results.

Statistical analysis

The two sets of homogeneous data from the repeated experiments were pooled together using the average pooling method. Also, the pooled data was transformed using the Square-root transformation method before the analysis. Normality testing was conducted using the Shapiro-Wilk test to evaluate whether our sample data followed a normal distribution. It revealed that the data did not significantly deviate from normality ($p > 0.05$), thus supporting the normality assumption for subsequent parametric tests. Additionally, Levene's test was employed to ensure homogeneity of variance across groups. The results of Levene's test indicated no significant difference in variances among the groups ($p > 0.05$), validating the assumption of homogeneity of variance and ensuring the reliability of our statistical inferences. Data were subjected to one-way analysis of variance (ANOVA) using the computer software Statistics (version 8.0, Analytical Software, Tallahassee, FL). Means were separated with Tukey's honest significant difference (HSD) at 1% probability. The values from the analysis were back-transformed and presented in Table 1.

RESULTS

From a comprehensive collection effort, eight *L. theobromae* isolates were obtained from the rotten avocado fruits sourced from the four markets. These isolates despite their diverse origins, exhibited similar morphological characteristics. The colony texture was fluffy, with pale white hyphae that later transitioned to a distinct dark brown. Immature pycnidiospores were aseptate, hyaline, subovoid to ellipsoid-oblong, thick-walled (appearing as double-walled), with a truncate or flat base. Mature pycnidiospores were dark, had one septation, and sometimes had longitudinal striations (Fig. 1).

BLAST analyses performed on the DNA sequences in the domain of NCBI showed the highest similarity (above 95%) to *L. theobromae*. Therefore, all the isolates were identified as *L. theobromae,* and the sequences were successfully submitted to NCBI GenBank under the accession numbers PP357451, PP373853 - 59. Phylogenetic analysis grouped the *L. theobromae* isolates into a single clade.

All eight *L. theobromae* isolates were found to be pathogenic and statistically similar in terms of virulence, with rotten area of fruit (RAF) values ranging from 20.32–21.89 cm^2 (HSD = 3.70). Three days after inoculation, the *L. theobromae*-inoculated exocarp of the fruits began to exhibit dark brown to black rot. As the fruit ripened, the rot spread from the initial little circular patch across the entire fruit. A similar symptom appeared on fruits inoculated on the stem end portion. A cross-section cut of the fruits showed a dark discoloration of the mesocarp and a hastened ripening. Controls (i.e., no inoculation and *F. oxysporum* inoculation) did not cause avocado fruit to rot after 96 h. The characteristics of the re-isolated fungi were similar to those described previously. Additionally, the *L. theobromae* plug inoculated at the stem end portion of healthy fruits resulted in disease-symptom expression similar to fruit rot.

The effectiveness of the bioagents in suppressing the growth of *L. theobromae* (PP373854) was evaluated *in vitro*. Mycelial growth of the tested fungus showed significant differences ($P < 0.01$) at all stages of the incubation period (Table 1). Except for garlic at 20 g/100 ml of water concentration, the rest could not retard entirely but suppressed the fungal growth. There was a significant difference ($P < 0.01$) in the effect of the bioagents on the mycelial growth rate (MGR) of *L. theobromae*. In the control set-up, which was not treated with any bioagents, the tested fungus had a MGR of 0.92 mm/h (Table 1). The least mycelial growth rate of the pathogen was recorded on PDA plates treated with garlic at 20 g/100 ml of water with no organism growth. The efficacies of both concentrations of garlic were significantly more effective in suppressing the growth of the tested fungus than black pepper and baking soda. Baking soda at 10 g/100 ml and 20 g/100 ml were not significantly different, with MGRs of 0.48 and 0.34, respectively (Table 1).

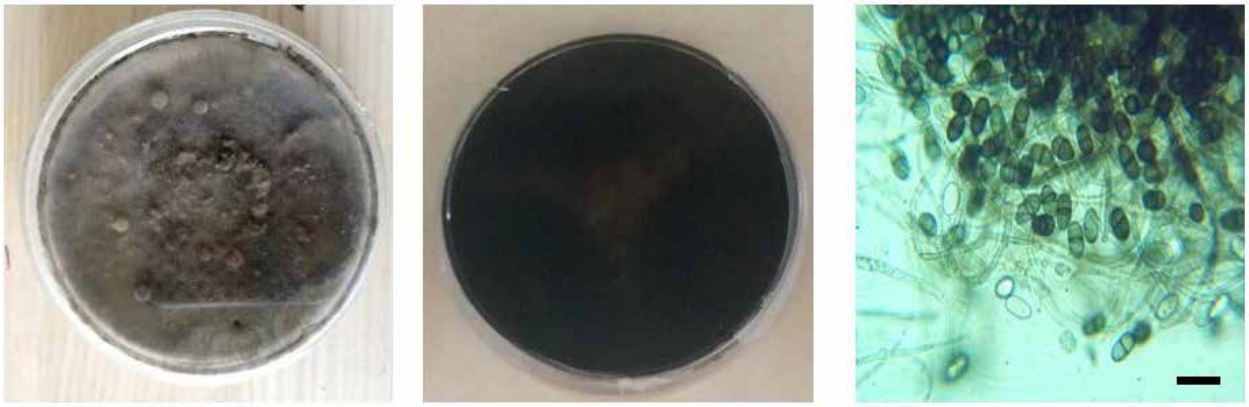

Figure 1. Morphological characteristics of the avocado fruit rot pathogen (*L. theobromae*)
Left: obverse view of *L. theobromae* on a plate; middle: reverse view of *L. theobromae* on a plate; right: conidia under a compound microscope (bar – 30 µm)

Table 1. Mycelial growth and MGR of *L. theobromae* isolate, PP373854, on bioagent-treated PDA with time

Treatments	Mycelial growth (mm) with time				MGR (mm/h)
	24 h	48 h	72 h	96 h	
Control	31.25±1.53^{a}	77.13±4.41^{a}	85.63±4.38^{a}	87.88±2.13^{a}	0.92±0.02^{a}
Black pepper 10	20.75±1.98^{b}	50.36±5.46^{b}	62.13±5.47^{a}	84.36±5.63^{ab}	0.88±0.06^{a}
Black pepper 20	23.86±1.91^{ab}	53.25±3.74^{b}	71.00±8.11^{a}	76.00±7.88^{a}	0.79±0.08^{ab}
Baking soda 10	9.62±2.89^{c}	21.50±3.51^{c}	30.00±5.30^{b}	46.00±6.74^{bc}	0.48±0.10^{bc}
Baking soda 20	5.00±0.59^{cd}	21.38±1.62^{c}	26.63±1.74^{bc}	32.63±1.80^{cd}	0.34±0.02^{cd}
Garlic 10	0.00±0.00^{d}	0.00±0.00^{d}	5.13±5.13b^{c}	7.75±7.75^{d}	0.08±0.08^{d}
Garlic 20	0.00±0.00^{d}	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{d}

Values are expressed as Mean± S.E. for four replicates per group. Values in the same column followed by different letters indicate that means are significantly different from each other according to Tukey's honesty significant difference (HSD) test at $p \leq 0.01$. MGR refers to the mycelial growth rate of *L. theobromae* on bioagent-coated PDA

DISCUSSION

Postharvest diseases remain a main quality problem when avocados are displayed for retail sale. Stem end rot and anthracnose are the most devastating diseases of concern after harvest worldwide (Story & Rudge 1997). In both situations, decay can spread deep into the flesh.

The cause of stem end rot disease is primarily attributed to *L. theobromae* and sometimes to *Dothiorella* spp. and *Phomopsis perseae* (Coates et al. 1997), remains a mystery. While the mode of infection has not been clearly established, several hypotheses have been put forward. These include the possibility of the fungi being endophytes of avocado stem tissue, gradually colonizing the inflorescence, fruit pedicels, and eventually the stem-end tissue, as observed in mango stem-end rot (Johnson et al. 1992). Another theory suggests that the fungal spores infect the avocado at flowering, leading to the colonization of the fruit stem-end tissue (Wanjiku et al. 2020). Lastly, there is a belief that rot fungi may infect at harvest time through the freshly cut surface of the fruit pedicel (Everett 1999).

The *L. theobromae* isolates showed in this study similar morphological, pathological, and genetic characteristics in the study and were obtained from all the visited markets. It confirms the assertion that *L. theobromae* is a cosmopolitan organism widely spread across the globe (Mohali et al. 2005).

The evaluation of the antifungal properties of baking soda and garlic showed positive results in this study. These bioagents have been widely employed to eradicate crop pests like fungi, bacteria, and insects. It has been reported that the active ingredient in garlic, allicin, is responsible for most of its biological effects, including bactericidal, antifungal, and antiviral properties (El-Saber et

al. 2020). Allicin is an organosulfur and inhibits mycelial growth and conidia germination of fungal pathogens. Our results agree with the finding of Hosseini et al. (2020), who recorded tremendous suppression of the mycelia growth of *Colletotrichum nymphaeae and Botrytis cinera,* rot pathogens of strawberry and tomato, using garlic. The active ingredient of black pepper, piperine, is an alkaloid that also suppresses fungal pathogens' conidia and mycelia growth (Alyaseen 2018; Osman et al. 2021). Although it suppressed the growth of the tested fungus, its bioactivity was lower than garlic and baking soda. Baking soda alters the pH balance of the fungi and thus prevents its proliferation (Afolabi et al. 2018). It could reduce the MGR of the fungus by 50%, which is significantly important for further studies in altering the concentrations to a higher level.

CONCLUSIONS

The isolates of *Lasodiplodia theobromae,* the causal agent of stem rot of avocado fruits at the post-harvest stage obtained from spoiled avocado fruits from 5 markets, showed similar morphological characteristics and virulent ability. The growth of *L. theobromae* mycelium was inhibited with garlic bulb powder and less with baking soda, which predicts their practical use in minimizing the occurrence of post-harvest deterioration of avocado fruits due to *L. theobromae*, providing a further study on fruit protection.

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Conflict of interest

The authors declare that there is no conflict of interest in this report.

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