In vitro antifungal efficacy of aqueous leaf extracts of neem (*Azadirachta indica* A. Juss.) against *Lasiodiplodia theobromae,* the cause of kola nuts rot in West Africa

***Ganiyu Shittu Olahan and Ibrahim Ajadi**

Department of Plant Biology, University of Ilorin, Nigeria
**Corresponding author's email: olahan.gs@unilorin.edu.ng*

Abstract

In vitro efficacy of eco-friendly aqueous neem (*Azadirachta indica* A. Juss.) leaf extract was explored against the fungal pathogen *Lasidioplodia theobromae* (Pat.) Griffon & Maubl., the cause of storage rot of kola nuts (*Cola nitida* Schott & Endl.), in West Africa. Aqueous neem leaf extracts at concentrations of 10%, 30% and 50% (w/v) were used to amend freshly prepared potato dextrose agar culture medium on which discs of *L. theobromae* were inoculated followed by incubation at room temperature for 72 hours. The findings demonstrated significant inhibition of fungal growth, with the highest inhibition observed at 50% (w/v) concentration of the extract. This suggests that aqueous neem leaf extract possesses fungicidal properties against *L. theobromae*, offering a natural alternative for controlling this pathogen and reducing pesticide residues in stored kola nuts, as well as its effects on consumers and the environment as a whole.
Keywords: *Azadirachta indica*, Disc diffusion method, Kolanuts, *Lasidioplodia theobromae*.

Introduction

Kola nuts, fruits of a perennial tree belong to the genus *Cola* in the Malvaceae family (Shamso and Khattab, 2016). West Africa is the home to approximately 40 species of *Cola*, with *Cola acuminata* and *C. nitida* being the two economically important species in the forest regions of West Africa and the Caribbean Island (Sousa and Raizada, 2020). In regions where kola nuts are cultivated, the harvesting process typically involves manually plucking ripe pods from the trees using a tool called go-to-hell, which is affixed to a long bamboo stick. Additionally, farmers gather pods that have naturally fallen to the ground. Subsequently, the nuts are cured and then sorted according to their sizes to facilitate their storage in large baskets. These baskets are firstly lined with thin transparent nylon sheets, followed by a layer of *Newbouldia laevis* (hup plum leaf) leaves positioned with their upper surface facing downwards, exposing the nuts to the back surface of the leaves (Oladigbolu *et al*., 2023). The nuts are meticulously layered inside the baskets with *Parkia biglobosa* (African locust bean leaf) leaves placed evenly on top of each layer. These baskets are then stored in conditions of typical room temperature (27 °C) and relative humidity.

Preventing disease outbreaks on kola nuts during storage remains a primary concern for farmers and traders, driving their ongoing efforts to find solutions. According to Kaur *et al*. (2023), a significant post-harvest challenge faced by kola nut farmers is incidence of storage rot, caused by *L. theobromae*. Severe outbreaks of storage rot, resulting in losses of up to 50%, have been documented (Xue *et al*., 2023). This disease-related challenge during storage significantly undermines the already limited production level achieved by kola nut farmers. Kola nut traders commonly utilize chemicals such as chlordane to safeguard their product from diseases. According to Aikpokpodion (2013), traces of chlordane were found in all kola nut samples collected from Oyo, Osun, and Ogun States in varying concentrations. Additionally, Olorunmota *et al*. (2021) highlighted that a significant proportion (88.3%) of kola nut traders opt for phostoxin (aluminium phosphide) to combat storage diseases, while 2.5% and 1.7% employ botanical methods and integrated pest management, respectively.

The widespread use of chemicals among kola nut traders for preservation during storage has raised significant concerns, particularly because the kola nuts are often consumed raw by humans. Although some of the chemicals used are recommended, Azeez (2015) found that all kola nut traders who utilize chemicals to control pests and diseases purchase them from vendors in the open markets where there is usually no standardization; expired or counterfeit products may be sold to illiterate farmers, the use of which pose serious threats to human health.

Currently, the most widely recognized safe control method for kola nuts during storage, as practiced by local farmers, is the periodic removal of diseased kola nuts during the storage period. Using natural plant products for biological control offers a promising alternative for managing plant diseases by offering eco-friendly sustainable alternatives to synthetic pesticides (Khan and Javaid, 2020). Moreover, the use of plant extracts can mitigate the development of resistance in pathogens, a common issue with chemical pesticides, thereby ensuring

long-term efficacy in disease management. Numerous studies have demonstrated the efficacy of various plant parts' extracts against *L. theobromae* (Dianda *et al*., 2020). Given the considerable potentials of plant parts in this regard, our study aimed to explore the *in vitro* antifungal properties of aqueous leaf extract of neem against *L. theobromae*, the prevalent post-harvest fungal pathogen affecting kola nuts.

Materials and Methods

Plant collection

Fresh neem leaves were collected from within the premises of the Cocoa Research Institute (CRIN), Headquarters in Ibadan, Oyo State, Nigeria. To remove dust and other extraneous objects, the leaves were washed under running tap water and then dried. The kola nut samples were obtained from Okuku market, which is located at latitude 8.01585^0 N and longitude 4.67249^0 E in Osun State, Nigeria.

Preparation of the aqueous leaf extract

The procedure of Mubayi *et al*. (2012) was closely followed in order to prepare the aqueous extracts of *A. indica* at 10%, 30% and 50% (w/v) concentrations. The extracts were prepared by weghing 10, 30 and 50 g of the leaves and properly washing them separately in distilled water. The leaves were then finely chopped and placed in a 250 mL Erlenmeyer flask containing 90, 70, and 50 mL of sterile distilled water, respectively. The mixture was boiled for 5 min and then filtered.

Preparation of culture medium

Potato dextrose agar (PDA) was used as the growth culture medium throughout the entire study. PDA (39 g in 1 L of distilled water) was prepared in accordance with the manufacturer's instructions. The suspension was homogeneously dissolved and autoclaved for 15 min at 121 °C and 103.4 kPa pressure.

Isolation and morphological identification of the test organism

Using a sterile knife on an ethanol-sterilized surface, tiny pieces of infected kola nut tissue revealing an expanding rot edge and adjacent healthy tissue were cut. The samples were cleaned and surface sterilized by soaking for 30 secs in 10% commercial off bleach Sodium hypochlorite (NaOCl) to remove surface contaminants rinsed three times in sterile distilled water for five minutes, and then blotted dry with sterile filter paper. Three pieces of the sterile infected tissues were plated on the PDA plates. For seven days, the plates were incubated at 27 ± 2 °C. Thereafter, pure colonies of the observed fungal isolates observed were maintained for examination according to Nakuleshwar *et al*. (2013). The fungal morphology was examined both macroscopically and microscopically. For the microscopic analysis, a compound microscope was used with a lactophenol blue-stained slide containing a small portion of the mycelium prepared, following the method described by Mahmut *et al*. (2023).

Molecular identification of the pure fungal isolate

Genomic DNA from the isolate was extracted using Quick-DNA™ Fungal/Bacterial MiniPrepKit protocol (Zymo Research Group, California, USA). The quantity and concentration of the extracted DNA were assessed using a Nano-Drop 2000c spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, Delaware, USA). Subsequently, the extracted DNA was forwarded to Inqaba Biotec, branch office located in Ibadan, Nigeria, for amplification and sequencing in both forward and reverse directions. The primers used to amplify the nuclear ribosomal DNA (rDNA) fragments of the isolates were the Internal Transcribed Spacer 1 (ITS1) with the sequence TCCGTAGGTGAACCTGCGG and ITS4 with the sequence TCCTCCGCTTATTGATATGC. In The forward and reverse sequences obtained after sequencing were aligned using the BioEdit v. 7.2.5 software. The sequences of the isolates were compared with those deposited in the National Center for Biotechnology Information (NCBI), using the BLAST tool: (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Sawant *et al*., 2023).

***In vitro* antifungal activity of the aqueous neem leaf extracts**

To investigate the impact of the extracts on the inhibition and sporulation of *L. theobromae*, the method described by Okigbo and Emeka (2010) was used. Using a sterile syringe and 15 ml of freshly sterilized PDA medium, 1 ml of each concentration (10, 30 and 50% w/v) of aqueous leaf extracts of *A. indica* was injected into 9-cm diameter Petri dishes containing freshly prepared PDA amended with an antibiotic (chloramphenicol) to prevent bacterial growth. With the aid of a sterile cork borer, mycelial discs of 8 mm diameter were removed from the growing edge of the mycelia of actively growing *L. theobromae* cultures. Each disc was inoculated at the centre of the PDA plates in three replicates. A control plate of PDA medium without aqueous neem leaf extract was also inoculated with the test fungus to serve as the control. The diameter of mycelial growth of the fungus on each inoculated plate was measured every 24 hours with a transparent ruler until the control plate was fully covered with fungal growth. All the inoculated plates were incubated at 27 ± 2 °C. Efficacy of the aqueous neem leaf extracts was recorded in terms of percentage inhibition, which was calculated using the formula:

$$\text{PGI (\%)} = \frac{\text{FC} - \text{FT}}{\text{FC}} \times 100$$

Where; PGI (%) = Percentage growth inhibition,
FC = Fungal growth on the control plate; FT = Fungal growth on the treated plates.

Results and Discussion

Identification of the pure fungal isolate

This was done on the basis of the cultural, microscopic, morphological and molecular features of the pure fungal isolate. Visual observation of the colony on PDA plates showed colour change from light grey after four to seven days of incubation (Fig. 1A and B) to black after 2 weeks of incubation (Fig. 1C). Microscopic observation of the conidia showed that the fungus produced oval, immature and mature conidia with some distinct features. The mature conidia were septate and thin cell-walled (Fig. 1D), while immature conidia were non-septate and thick cell-walled (Fig. 1E**)**.

Sequencing comparison of the DNA obtained from the pure fungal isolate revealed that it was 100% identical to *L. theobromae* (MF114110) with 100% query cover in the GenBank database. According to Raja *et al.* (2017), dependable identification of fungal species through molecular techniques requires that the sequence comparison achieve specific thresholds when using the Basic Local Alignment Search Tool for Nucleotides (BLASTN) on the NCBI database. These thresholds include attaining a query cover of at least 80%, which ensures that a significant portion of the query sequence aligns with the database sequence. Additionally, the percentage identity must be at least 97%, indicating a high degree of similarity between the sequences being compared, thereby authenticating the identification in this study. PP732654 was the NCBI accession number assigned to the pure fungal isolate.

***In vitro* antifungal activity of the aqueous neem leaf extract**

The research investigated the inhibitory effect of neem leaf extracts at 10%, 20%, and 50% (w/v) concentrations on *L. theobromae* growth. Findings showed that the inhibitory effect increased progressively with higher concentrations, demonstrating the extract's fungicidal potential by effectively inhibiting fungal growth at low concentrations. After 24 h, inhibitions were 22.4%, 38.6%, and 54.4% for 10%, 20%, and 50% concentrations, respectively, which further increased to 34.6%, 49.5%, and 67.2% after 72 h (Fig. 2). Conversely, the control group displayed continuous fungal growth throughout the incubation period, underscoring the neem leaf extract efficacy. Many earlier scientists have reported antifungal properties of various plant species and stressed the significance of plants as probable sources of natural fungicides (Ferdosi *et al.,* 2022; Javaid *et al.,* 2022; Naqvi *et al.,* 2023). Khan *et al.* (2020) reported that soil amendment with leaves of neem markedly suppressed incidence of collar rot of chickpea caused by *Sclerotium rolfsii.* Similar effect of neem leaves was also reported by Sana *et al.* (2015) against southern blight disease of chili. These results are consistent with previous reports by Ablormeti *et al*. (1970), Dele and Abiodun (2015), and Dianda *et al*. (2020), who also reported that neem extracts effectively inhibited the mycelial growth of *L. theobromae*. Moreover, the effectiveness increases with higher extract concentrations. According to Ilondu (2011), *Carica papaya* and *Chromolaena odorata* exhibited fungicidal and fungistatic effects on *Botrylopodia theobromae.* Similarly, Sahi *et al*. (2012) found that garlic and neem showed inhibitory effect on *L. theobromae*.

The recognition that higher plants contain antifungal compounds has long been established as crucial for disease resistance (Miller *et al*., 2022). These compounds, which are both eco-friendly and biodegradable, are highly valued for managing plant pathogenic diseases (Kumar *et al*., 2021). Utilizing naturally occurring chemicals that inhibit the growth or reproduction of plant pathogenic fungi offers a practical and ecologically sustainable approach to integrated plant disease management. This approach is expected to play a significant role in the creation of commercial pesticides for crop protection (Tudi *et al*., 2021). The prohibition of certain synthetic pesticides has driven research into new pest and disease control strategies for kola nut in Nigeria. To reduce pesticide residues in marketable products like kola nuts, researchers and chemical companies are now focused on developing plant-derived pesticides with biological activity for crop protection (Oshatunberu *et al*., 2023).

Conclusion

The study highlights the significant biofungicidal potential of aqueous neem leaf extracts against *L. theobromae*, particularly at a concentration of 50% (w/v). This natural extract demonstrates strong fungicidal properties, providing an effective alternative to conventional chemical fungicides. The use of neem leaf extract can help control fungal infections in kola nuts, ensuring safer storage and consumption by reducing pesticide residues.

Contribution of authors

The study was undertaken by IA as his undergraduate project under the supervision of GSO. The manuscript was prepared jointly.

Conflict of interests

The authors declared no conflict of interest.

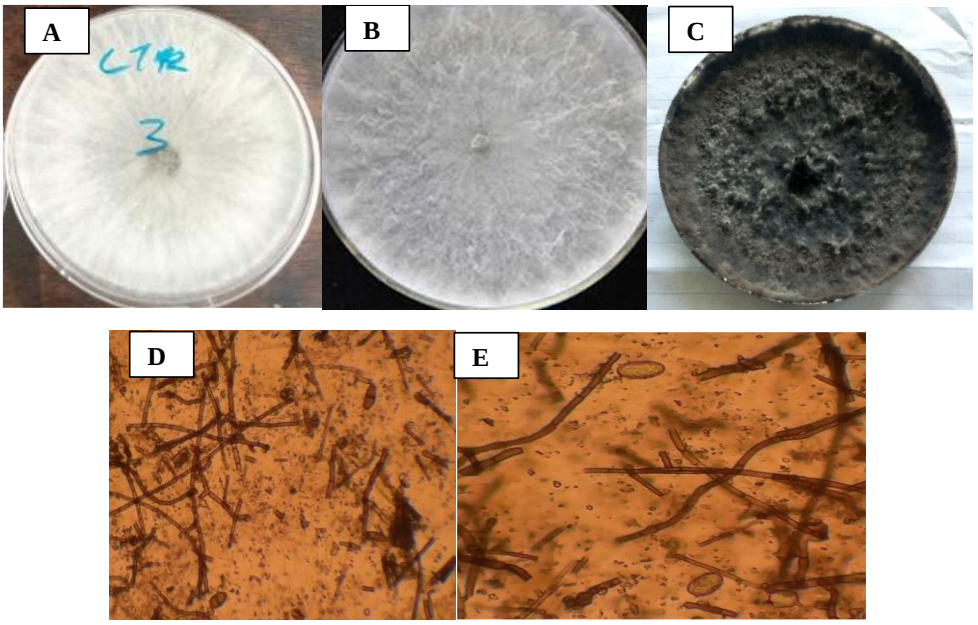

Fig. 1: Morphological characters of *Lasidioplodia theobromae*.

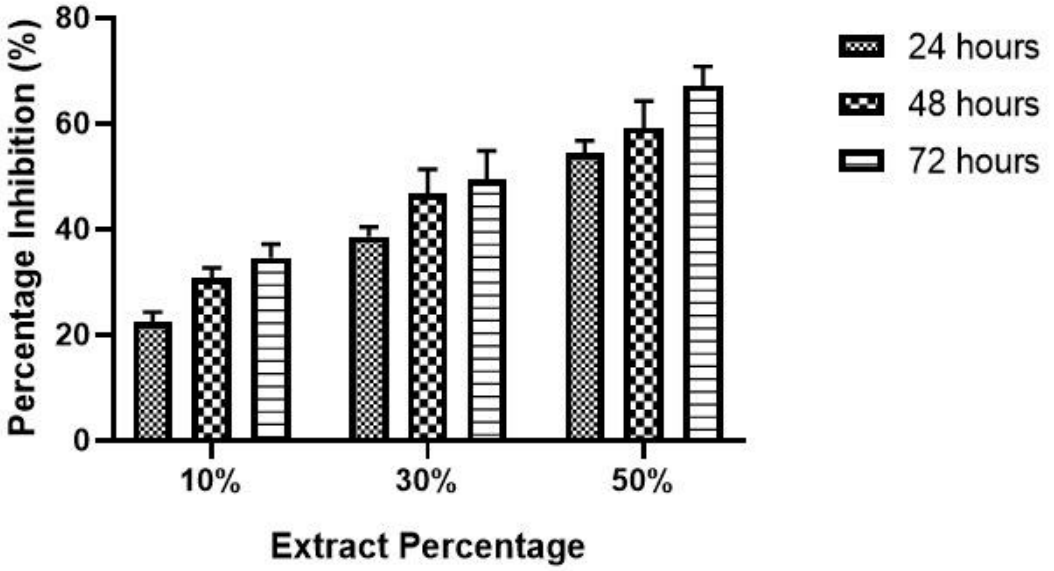

Fig. 2: Effect of *Azadirachta indica* leaves extract at 10, 30 and 50% concentrations on *Lasidioplodia theobromae* mycelial growth.

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