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Mycoflora of *Annona muricata* L. (Soursop) Fruits

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Abstract:- *Annona muricata* L. (soursop) fruits are susceptible to fungal pathogens especially during post-harvest handling and storage and this leads to low fruit quality. In this study, we identified some fungi associated with soursop fruits using molecular tools. The internal transcribed spacer (ITS) region of the fungal 16S rRNA gene was amplified using a Polymerase Chain Reaction (PCR), and the reaction product was then sequenced to determine the identity of the fungal species. The isolated fungi were identified by a blast search on the National Center for Biotechnology Information (NCBI) as *Lasiodiplodia pseudotheobromae*, *Aspergillus niger* and *Aspergillus homomorphus*. An evolutionary tree was constructed by the composite likelihood method to display sequence relationship and common ancestries. The isolated fungal pathogens are capable of producing harmful mycotoxins. The occurrence of these fungi can lead to huge economic losses, thereby posing a potential threat to soursop fruits in Nigeria.

Keywords:- *Annona muricata, Fungi, Microorganisms, Diseases, Sequencing, Phylogeny*

I. INTRODUCTION

Fruits are rich in protein, water, dietary fibre, and vitamins for humans especially in the fight against nutrition-related chronic diseases (Djellout et al., 2020). Ziv and Fallik (2021), highlighted the full health benefits of an increase consumption of fruits which can lead to a lower risk of heart disease and cancer. Post-harvest losses in fruit production (soursop) continues to pose a threat for modern agriculture from their production through harvest up-until storage. Fruits which represents a vital source of nutrients for human consumption are liable to contamination by microbial pathogen that occur majorly at the post-harvest handling stage before it gets to the consumer. Post-harvest diseases are major causes of fruit spoilage and deterioration up to 50% of the entire fruit, with fungi invasion ranging up to 70% (Hernandez-Guerrero et al., 2020). Generally, fungal organisms are responsible for the changes in fruits during storage for e.g. rot appearance that leads to a reduced shelf life of fruits (Hozbor et al., 2006; Djellout et al., 2020). Interestingly, these fungi often survive with the help of the stored nutrients found in the fruits (sugar, protein, lipid, and vitamins) (Veld, 1996; Ife & Bas, 2003; Djellout et al., 2020).

Annona muricata L. (soursop) of the family Annonaceae is a multipurpose fruit with global recognition due to its nutritional values (Adeola & Aworth, 2010; Moreira et al., 2018). However, it has been reported to be highly susceptible to microbial attack during storage and this results in the loss of fruit quality, quantity and availability, thereby leading to severe economic losses (Hasan & Zulkahar, 2018; Ntsoane et al., 2019). Hypothetically, researchers affirm that microbes living in the tropics are diverse, and poor characterization with wrong microbial sampling can hinder sound understanding of their role thereby hampering the discovery of novel species (Pinto et al., 2014). Thus, early detection is paramount in preventing disease spread with minimal damage to fruit crop (Yang et al., 2013).

In order to understand the diverse nature of microorganisms, a number of traditional and modern techniques for the identification and detection of novel pathogen have been discovered (Handelsman, 2007). With the help of Polymerase Chain Reaction (PCR) based methods (culture-dependent and culture-independent), Sanger sequencing and bioinformatics, characterization and identification of microorganisms with hidden diversities are more revealed practically (Gibbs & Mackenzie, 1997; James et al., 2006).

This research was carried out to identify and isolate the fungal micro-organism associated with the spoilage of *Annona muricata* fruits in storage. By performing an experiment that extracts the DNA of the microbes the study intends to provide valuable information of their impact on crops and human health.

II. MATERIALS AND METHODS

A. Study Area and Sample Collection

Annona muricata L. fruits with disease symptoms were sampled from local markets in Port-Harcourt and Obio-Apkor Local Government Areas in Rivers State, Nigeria. The fruits were placed in polythene bags and transported to the Regional Centre for Biotechnology and Bio-resources Research (RCBBR) Laboratory, University of Port Harcourt, Choba, Rivers State, Nigeria. Fruits were washed with distilled water to eliminate endophytic organisms before culturing. Purification and sequencing of the PCR products were done at the International Institute for Tropical Agriculture (IITA), Ibadan. The *Annona muricata* fruit used in the study is presented in Figure 1.

B. Isolation of Fungi from Annona muricata Fruits

Fungal organisms were isolated from matured soursop fruits, with visible damage and signs of diseases. Segments of the affected area or tissues of the diseased fruits, were cut out using a sterile scalpel and treated with a 1% sodium hypochlorite solution for 3min, washed with distilled water, and then placed in the centre of Petri dishes with potato dextrose agar (PDA). Petri dishes were incubated for seven

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days with proper observations of colour, texture, and colony formation (Mulkay et al., 2010). Frequent re-isolations were performed to preserve the purity of the strains. Freshly made PDA was used to grow the uncontaminated (pure) isolates, incubated at 28±2℃ for seven days, and then stored at 4℃ prior to DNA extraction.

C. DNA Extraction, PCR, and Sequencing

Genetic material (DNA) was obtained using the protocol of Quick-DNA^{TM}Fungal/Bacterial MiniPrep Kit (Zymo Research Group, California, USA) as described by the manufacturer. Fragments of the nuclear ribosomal DNA (rDNA) was amplified using fungal universal primer pair ITS4, forward (5´-TCCTCCGCTTATTGATATGS-3´) and ITS5, reverse (5´-GGAAGTAAAAGTCGTAACAAGG-3´) (White et al., 1990).

Amplifications were performed in a thermocycler (Eppendorf) subsequently with a first step in denaturation (94℃) for 5 minutes; 36 cycles of denaturation (94℃) for 30 seconds; annealing (54℃) for 30 seconds and elongation (72℃) for 45 seconds, with a final extension (72℃) for 7 minutes. The amplified PCR products were subjected to gel electrophoresis using 1.5% agarose gel in TBE 1X, stained with ethidium bromide (13µL/50ml) for 40 minutes at 100volts. At the end of the run, the gel was photographed under UV using a Gel Documentation System (Gel Documentation microDOC^{TM}, Cleaver Scientific Ltd, UK).

Amplified products were sequenced using Sanger sequencing technology [GeneAmp$^{(R)}$ 9700 PCR System (in 9600 emulation mode), Applied Biosystem, California, and USA]. The resulting sequencing were subjected to BLAST search on NCBI database to reveal the species identity of the isolates. ITS sequences were then deposited in GenBank of NCBI database to retrieve accession numbers.

D. Phylogenetic Analysis

To obtain high quality reads, raw sequences obtained were cleaned and edited on Molecular Evolutionary Genetics Analysis (MEGA) software, version 7.0. MEGA was used to represent and build the evolutionary tree (Kumar et al., 2016) using the composite likelihood method to show the relationship among the sequence and their common ancestors (Hall, 2013).

III. RESULTS

A. Study Area and Sample Collection

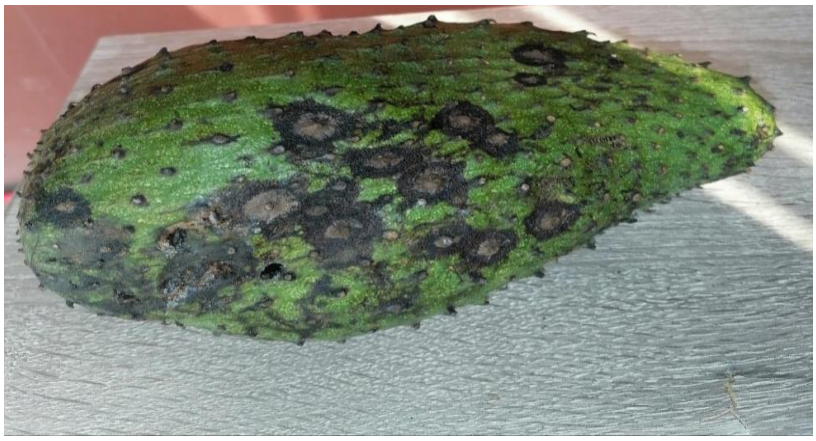

Fig 1 *Annona muricata* fruit with Diseased Symptoms

B. Fungal Organisms Associated with Annona muricata Fruits

The isolated fungi initially produced a thin mycelium which covered the entire 9 mm plate within three days. The mycelium with colonies grew fluffy within 5 days (see Fig. 2). Overtime, the pure culture produced a greyish black colonies within seven days as shown in Fig. 2.

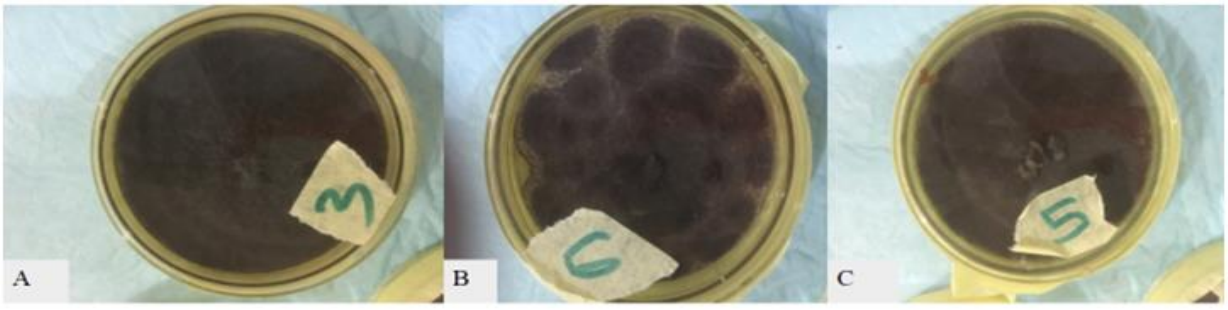

Fig 2 Pure Culture on Potato Dextrose Agar at 27+2℃ Isolated from *Annona muricata* Fruits: (A) Sample 3, (B) Sampe 4, and (C) Sample 5

➢ *DNA Concentration and Purity*

The concentration and purity of extracted DNA met the quality check for other experiments necessary for fungi characterization (Table 1).

Table 1 DNA Concentration and Purity of Fungal Isolates

Sample ID	DNA Concentration (ng/ µl)	DNA Purity at 260/280
3	16.97±0.88	1.66±0.03
4	46.27±7.43	1.91±0.02
5	56.85±0.12	1.89±0.02

C. PCR and Gel Electrophoresis

The amplified DNA from the fungal samples showed disease symptom with DNA bands pattern produced on agarose gel electrophoresis (Figure 3).

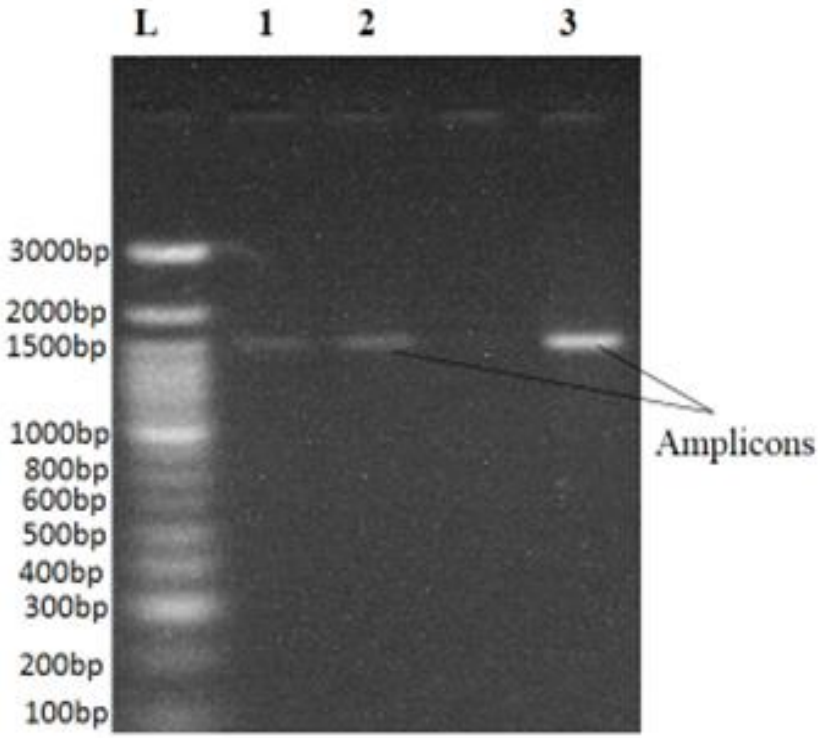

Fig 3 Gel Electrophoresis of Amplified Genes Generated from Fungul Isolates of *Annona muricata*

D. Molecular Characterisation of Fungi Associated with Annona muricata

Amplified genes were blasted against known species on NCBI database. The fungal isolates were identified as: *Lasiodiplodia pseudotheobromae* (sample 3), *Aspergillus homomorphus* (sample 4), and *Aspergillus niger* (sample 5). Sequences were submitted on Genebank and were assigned accession numbers: OM269043; OM273169; and OM269119 respectively. The taxonomic affinities of ITS sequences of isolates on Genebank are represented in Table 2.

Table 2 Taxonomic Identification of Fungal Organisms with Accession Number on GeneBank

Sample ID	Strain No	Blast identity	Percentage similarity	Accession Number
3	RCBBR _AEAOV3	*Lasiodiplodia Pseudotheobromae* (MH716405.1)	92.65%	OM269043
4	RCBBR _AVEAOV4	*Aspergillus homomorphus* (KJ888832.1)	98%	OM273169
5	RCBBR _AVEAOV5	*Aspergillus niger* (KU243044.1)	99%	OM269119

E. Phylogenetic Analysis

The phylogenetic tree constructed showed the evolutionary relationship between the fungal isolates and other fungi on Genebank. The sequence analysis showed genetic diversity between the isolates at each taxonomic level based on the alignment of their 18S rRNA gene sequences as presented in Figures 4-6. The numbers at each phyletic line (vertical line) represent the distance between each organism on the tree node. The alignment scores of both samples distribution of top 100 blast hits on 100 subject with red lines are presented in figures 7.

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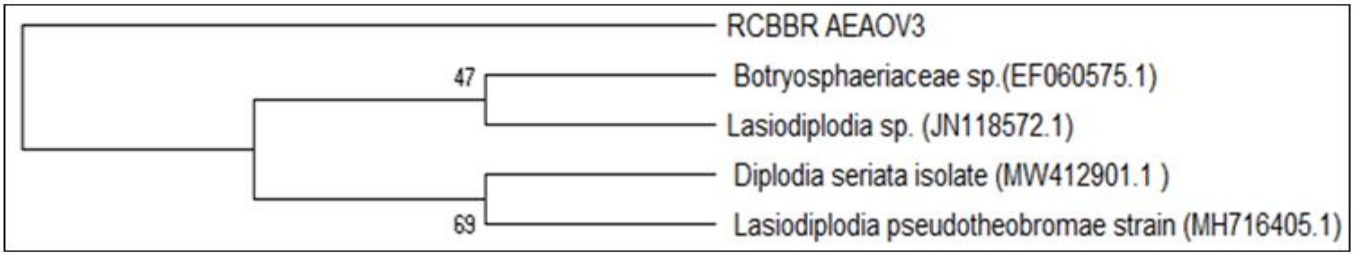

Fig 4 Phylogenic Tree of RCBBR _AEAOV3 Generated by Composite Likelihood Based on the ITS Gene Sequences

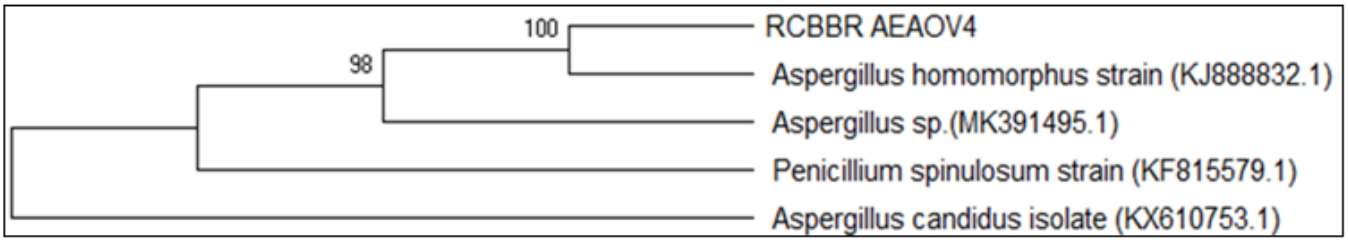

Fig 5 Phylogenic Tree of RCBBR _AEAOV4 Generated by Composite Likelihood Based on the ITS Gene Sequences

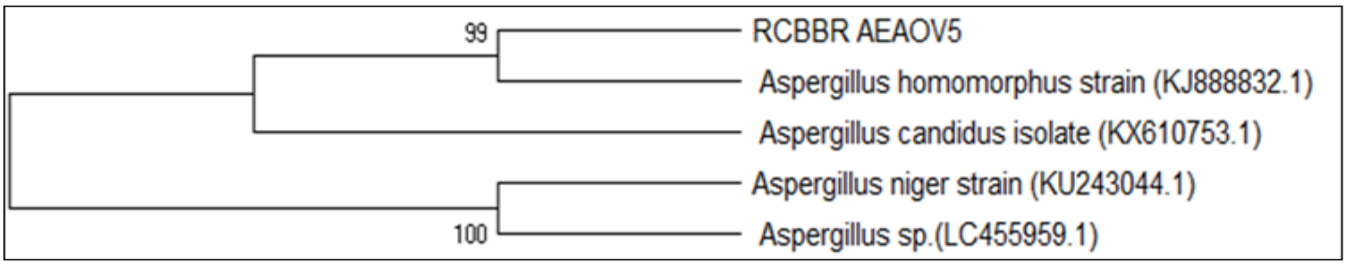

Fig 6 Phylogenic Tree of RCBBR _AEAOV5 Generated by Composite Likelihood Based on the ITS Gene Sequences

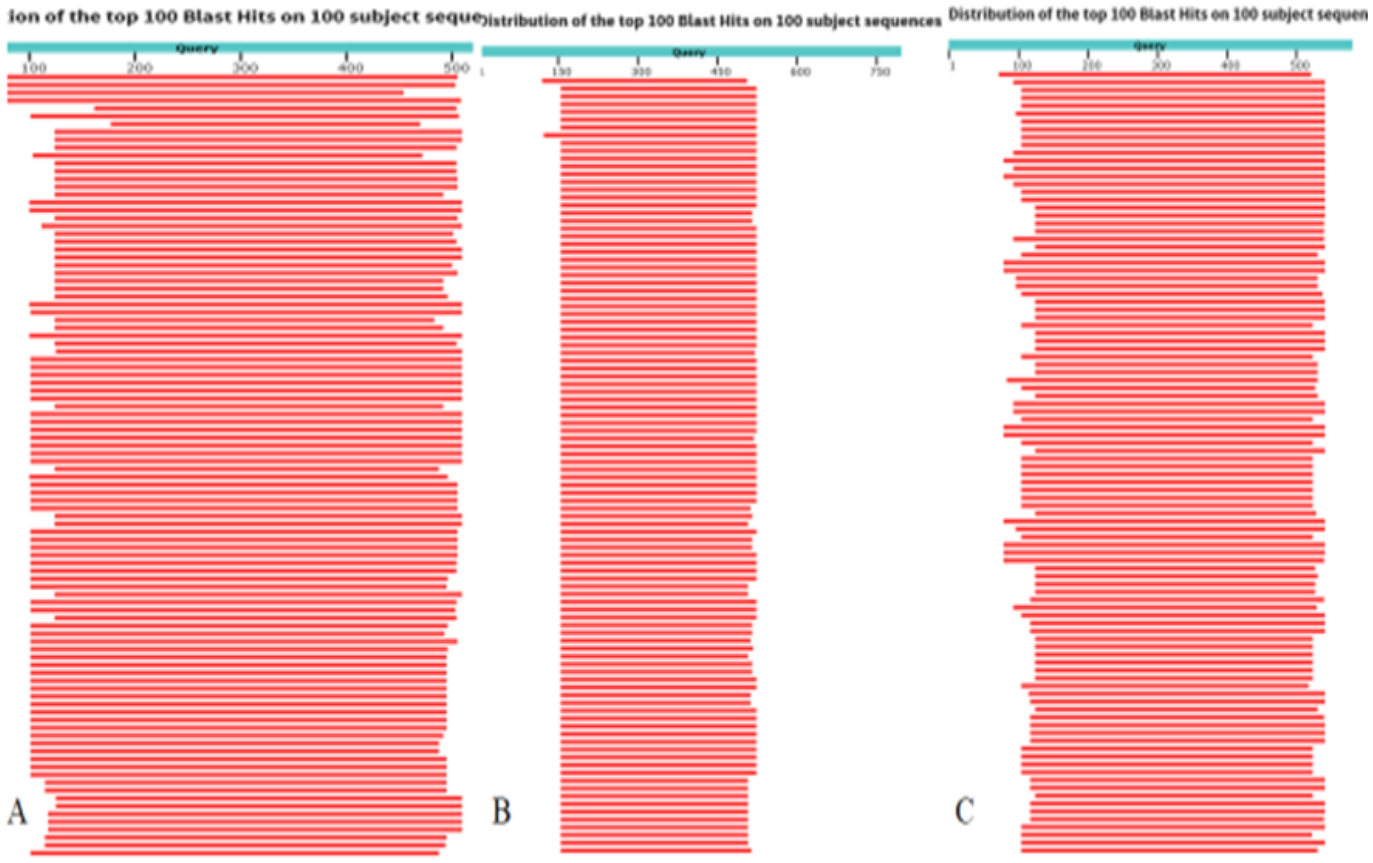

Fig 7 Alignment Scores of Aligned Sequences (A) RCBBR _AEAOV3 (B) RCBBR _AEAOV 4 (C) RCBBR_AEAOVS

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IV. DISCUSSION

A number of fungal organisms that produce rots and deterioration on *Annona muricata* fruits during post-harvest storage were revealed: *Lasiodiplodia pseudotheobromae, Aspergillus homomorphus* and *Aspergillus niger*. The genera *Lasiodiplodia* and *Aspergillus* are known to invade and penetrate plant tissues, producing rots of different types in storage and secrete mycotoxins which result in food poison in humans after consumption (González-Ruíz et al., 2021).

Species of *Lasiodiplodia*, of the family Botryosphaeriaceae are widely known to cause loss in tropical plants (Phillips et al., 2013). The fungus is known as a key pathogen that poses concern for agriculture due to the post-harvest rot and other diseases on fruits (Sakalidis et al., 2011; Ismail et al., 2012; Marques et al., 2013; Zhang, 2014; Dissanayake et al., 2015). Alves et al. (2004) reported that the impact of the disease can lead to bark discolouration, death of the plant and decline in production resulting to significant economic loss. Spread of the disease is through wound cut on the plant which makes it easier for the pathogens to penetrate (Liang et al., 2020). Successfully, the pathogen have been identified in over 52 plants across the globe (Mehl et al., 2017; Sudarma et al., 2022). The fungus has been found to secret enzymes of different types and naturally, high temperature can increase its growth (Sudarma et al., 2022). When compared to other genera, Lasiodiplodia was shown to be one of the highest and most frequent post-harvest damages in soursop species, according to González-Ruz et al. (2021).

According to Pipattanapuckdee et al. (2019), *Lasiodiplodia pseudotheobromae* is an opportunistic pathogen with a latent endophytic stage that can cause a wide range of illnesses, especially when subjected to stress or unfavorable environmental conditions. *Lasiodiplodia pseudotheobromae* was reported as the most damaging post-harvest diseases of mango and several fruit trees, resulting in losses up to 40% (Alam et al., 2021). Records of previous research showed *L. pseudotheobromae* as a highly diverse fungus that has a large range of hosts, especially from the tropics and subtropics (Picos-Munoz et al., 2015). Thereafter, Cabrera et al. (2022) revealed that *L. pseudotheobromae* was to blame for the regressive mortality of both an agricultural crop and an ornamental plant. Thus, *L. pseudotheobromae* is capable of causing rot and final death in agricultural crops.

The genus Aspergillus is a significant filamentous species responsible for a variety of plant and food diseases, as well as the potential buildup of mycotoxins (Perrone et al., 2007). Due to how they appear under a microscope, *Asperigillus* got their name from the Latin word *"Aspergillum,"* which roughly translates to "holy water sprinkler." *Aspergillus homomorphus*, a filamentous fungus of the genus *Aspergillus* that belongs to the section Nigri and was first reported in 1995 (Samson et al., 2004), is well-known for producing harmful mycotoxins. The most significant fungi responsible for food spoilage and bio-deterioration of materials are found in *Aspergillus* section *Nigri* (Gams et al. 1985), also known as black aspergilli. They have been used extensively for various biotechnological purposes, including production of enzymes and organic acids (Samson, et al., 2004, Varga et al., 2011).

Futyma et al. (2019) stated that *A. homomorphus* is very important pharmaceutically and industrially producing useful secondary metabolites. *A. homomorphus* strain was isolated from millet (*Pennisetum glaucum* L.) grain and thus, showed that the species is not limited to fruit nor soil (Steiman et al., 1994; Borrego–Terrazas et al., 2014; Hussein et al., 2017). *A. homomorphus* was reported as part of a group of opportunistic fungi that b virulent when the immunity of the host is compromised and a major pathogenic agent, due to its tolerance to a wide range of environmental conditions (Tan et al., 2022). Despite that, several plants provide nutrients suitable in the fight against *Aspergillus* infections, the species poses a life threat to the society especially in immuno-compromised patients.

With 15 different strains with black conidia across the genus, *Aspergillus niger* is the most prevalent plant pathogen in the genus *Aspergillus* (Raper & Fennell, 1965). *Aspergillus niger* is known to contaminate food and is also the agent behind the "black mould" that appears on the peels of some fruits and vegetables, including grapes, apricots, onions, and peanuts, among others. Palencia et al. (2010) revealed *A. niger* as a potential mycotoxin producer having adverse effects on plants and humans as pathogens. Afterwards, it was found that *A. niger* was a significant plant pathogen that caused white yam to produce fumonisin B2 mycotoxin (Dania et al., 2021). According to reports, *Aspergillus niger* causes a number of post-harvest diseases, such as mango rotting, stem rot of dracaena, black mold rot of cherries, kernel rot of maize, fruit rot of grapes, fruit rot of bananas, rot of tomatoes, and fruit rot of grapes, which cause discoloration, quality degradation, and a decrease in the commercial value of these crops (Sharma, 2012; Martinez et al., 2021; Nguyen et al., 2023). For many plants, *A. niger* is a severe disease that can cause rotting, decomposition, and plant death (Tawfik et al., 2022). According to Tawfik et al. (2022), *A. niger* is a dangerous plant pathogen that causes mycotoxins (ochratoxins and aflatoxins) to be secreted and produced, which contaminate various fruits.

However, *Aspergillus niger* has been utilized for many years in the gluconic acid and citric acid manufacturing processes, which are both common food preservatives found in canned fruits, shampoos, and blood preservatives (Sharma, 2012). A study reviewed *Aspergillus niger* as an endophytic fungus in the host plant tissues without causing symptoms of infection in the host (Lubna et al., 2018). Thus, *A. niger* can survive in nature in every plant species. According to Lubna et al. (2018), endophytic fungi, such as *A. niger*, are the primary source of naturally occurring bioactive chemicals with potential uses in the food sector. Endophytes can therefore produce bioactive substances that are comparable to those made by their host plant (Zhao et al., 2010). In spite of the ubiquitous nature of *A. niger*, it occupies a wide habitats in animal and plant environments

and it is economically important both as harmful and beneficial microorganisms (Yu et al., 2021).

Wang (2016) suggested, that the presence of diseases cannot be disregarded as control measures should be carried out in the early stage of discovery of a disease, as to avoid significant economic losses in forestry and agriculture. Additionally, although *Aspergillus niger* and *Aspergillus homomorphus* seems to be less frequent or limited, their pathogenic expressions on fruit crop or plant products as myco-toxins and their association with different species contaminating foodstuff influences the need for further studies of the black aspergilli associated with fruits. Furthermore, training programs should be encouraged to educate sellers and the general populace on the challenges of contaminations of fruits and foodstuffs by fungal organisms.

V. CONCLUSIONS

The different *Aspergillus* and *Lasiodipodia* species isolated from *Annonna muricata* fruits are of tremendous economic importance and provide a significant risk of contaminating human health as well as a financial loss. This study on the fungal organisms associated with *Annona muricata* in Nigeria and its diseases occurrence provides reference value that are reliable in evaluating potential exposure risk of the population to these contaminants. Thus, the presence of these pathogens on fruits cannot be ignored as control measures that involve close monitoring should be put in place at the early stage to avoid the extreme spread of potential disease.

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