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RESEARCH ARTICLE

Molecular characterization of fungi producing aflatoxin in *Vigna subterranean* (Bambara Groundnut) sold in three selected markets in Enugu Metropolis

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ABSTRACT

Humans require nutritious food for a healthy diet. Bambara groundnut (*Vigna subterranean*) as a crop has many varieties of uses. However, toxic strains of *Aspergillus* species found in contaminated BG produce carcinogenic aflatoxins. Intake of food that has contamination of aflatoxin (>20 ppb) is unsafe. This study identified the fungi that produce aflatoxin in BG using a molecular approach. Samples were acquired from five different locations in Abakpa, New Main, and Ogbete Market in Enugu State, Nigeria. Streptomycin was used to inhibit bacterial growth, and the samples were directly plated on Saboraud Dextrose Agar to isolate the fungi. The plates were stored at room temperature for 48-72 hours and pure fungus culture was obtained by repeated subculturing. BG aflatoxin quantification was carried out using an enzyme-linked immunosorbent assay. The isolates were subjected to an aflatoxigenic analysis using molecular methods. The number of isolated fungi was 77 isolates. Among the isolates, the frequency of occurrence ranged from *Alternaria macrospora* and *Scolecosporae* (1% each) to *Aspergillus flavus* (32%). Aflatoxin levels in the samples were higher than the permissible threshold (20 ppb), ranging from 69 ppb (Abakpa) to 80 ppb (Ogbete). All sequenced and screened isolates contain an aflatoxin regulatory gene. The study results show aflatoxin contaminated BG samples, rendering them unhealthy for human consumption.

Keywords: *Aphis fabae, Tephrosia vogelii*, Phytochemistry, Repellency activity, LC-MS analysis.

INTRODUCTION

The use of agricultural food commodities has a number of downsides that have an impact on global food security (Ogwu et al., 2018). Humans require nourishing food that contains necessary nutrients to maintain a healthy diet. Proteins are required for structure (growth, tissue repair), while lipids and carbohydrates are required for energy (Yann et al., 2019). Bambara groundnut and cowpea, which are consumed throughout Africa, are high in these components. Foods (cereals, tubers, legumes, vegetables, etc.) can be contaminated by a variety of contaminants, including mycotoxins which are secondary metabolites that are deleterious to humans and animals (Ekpa et al., 2019). The toxic substances obtained from the genera *Aspergillus* (A), *Penicillium* (P), and *Fusarium* (F) produce mycotoxins (Yann et al., 2019).

Aflatoxins which are difuranocoumarin molecules produced through the polyketide pathway are highly stable and they can resist standard food processing (Peles et al., 2019). Aflatoxins have been found in oilseeds, barley, and wheat, among other foods. In both animals and humans, aflatoxins have been related to immunological suppression, they are the most dangerous of all mycotoxins, inflicting damage to the human body and can also aggravate kwashiorkor and stunted growth in children (Awuchi and Ogueke, 2021). They can induce stunting in children by suppressing cell-mediated immunity. Several studies have found that aflatoxins hasten the progression of HIV infection to AIDS (Wan et al., 2022).

Bambara groundnut (BG) is an unexploited, under-studied native orphan crop produced across Sub-Saharan Africa with tremendous potential. It's rich in nutrients and drought-resistant. *Vigna subterranea* can be cultivated without the use of fertilizers, which are sometimes expensive and difficult to get (Ogwu et al., 2018). In Nigeria, it is an essential leguminous crop, believed to have originated in Nigeria, particularly between Yola and Jos. It is varyingly referred to as Okpa in Igbo region of Nigeria; Kwaruru or Gurjiyain in the Hausa region of Nigeria; and Epa-Roro in the Yoruba region of Nigeria (Isadeha, 2018). Bambara groundnut is gaining popularity in a variety of food applications thanks to its favourable nutritional profile, particularly its high protein content. The most prevalent kind of BG product "okpa" is consumed by millions of Nigerians, making it one of the most common delicacies for both the rich and the poor. In general, "okpa" is enjoyed by almost every Nigerian, and as a result, it enjoys widespread popularity among people of various ages, genders, and socioeconomic positions. The patronage of "Okpa" is particularly prominent among travellers, students, and families (Aroh, 2018).

However, many people are exposed to food poisoning resulting from mycotoxins ingestion. Good hygiene in food handling and basic hygiene practices are often neglected by the populace. These to a greater extent have posed a serious threat to human health. Despite the numerous contributions of Bambara groundnut to the human diet and its economic importance, the plant has received little scientific attention. Hence, there is a need to characterize the aflatoxigenic fungi present in BG using molecular techniques thereby creating awareness of possible fungi contamination.

MATERIALS AND METHODS

Sample collection and preparation

Fifteen samples of *Vigna subterranean* were purchased from Abakpa, New market and Ogbete market, Enugu State, Nigeria, and transferred in an aseptic condition and transferred to the Microbiology laboratory in Lead City University, Ibadan for analysis. The samples were ground into powder using a clean Iwatani Blender, IFM-800 (Made in Japan).

Preparation of culture medium for fungal enumeration

19 grams of HImedia Saboraud Dextrose Agar (SDA) was dissolved in 500 mL of water, and homogenized by boiling for 15 minutes. The solution was sterilized using an autoclave at 21oC for 15 minutes and allowed to cool, and 1% of streptomycin sulphate was added to prevent the growth of bacteria.

Sterilization of materials

Glasswares were cleaned by washing them with detergent, then rinsed and covered with aluminium foil. The media used were also sterilized by autoclaving for 15 minutes at a temperature of 121°C. Inoculating needles and Scalpels were sterilized by immersing them in 70% ethanol and heating them in flames.

Fungi Isolation and preservation

One gram of each thoroughly mixed ground sample was weighed and mixed with 100 mL of sterile distilled water to give the mother inoculum. Serial dilution was performed for each sample by transferring 1 mL of each of the mother inoculum

into their respective 9 ml of sterile distilled water to make one in ten dilutions i.e.10^{-1}. 1 mL of the mixture from 10^{-1} was used to make 10^{-3}, and 1 mL was again used from there to make 10^{-5} dilution. The pour plate method was used for the fungi enumeration. 1 ml each of the dilution of 10^{-1}, 10^{-3}, and 10^{-5} was transferred into Petri plates using the pipette, after which 15 mL of the prepared sterilized molten PDA medium was poured into the petri dishes containing the serial dilutions, an were homogenized, kept at room temperature for 72 h. In the end, visible growths were subcultured into new medium, and was repeated to obtain a pure culture. 15 mL of the molten PDA was also poured into McCartney bottles, autoclaved and kept in a slanted position until it solidified. For preservation

Morphological identification of the fungi

The morphology of the isolates grown on the petri dish was observed and characterized according to the formation of spore, colour, hyphae and shape. Microscopic examination was performed by transferring the culture on the plate to a microscope slide using a sterile needle. A drop of cotton blue-in-lactophenol was used to stain the culture on the slide after which the slide was gently teased and then covered using a coverslip. The covered slide was then viewed under the 10x and 40x magnifications of the light microscope. The isolates were identified by comparing their characteristic.

Quantification of the Aflatoxin Load of the Bambara Groundnut

The aflatoxin content of the Bambara groundnut was measured using AgraQuant® Total Aflatoxin Assay 4/40.

Aflatoxin quantification procedure

The Bambara groundnut was ground to obtain representative samples. 20 g of the ground sample was transferred into a cleaned jar containing 100 mL of 70/30 (v/v) methanol/water extraction solution in a 1:5 ratio, and then the jar was tightly sealed. This was shaken and thoroughly mixed after which it was allowed to stand for 3 minutes. After three minutes of standing, the sample extract was filtered using Whatman #1 filter paper. The filtrate was used for the aflatoxin assay following the procedures of Jager & Tonin (2012).

DNA extraction

DNA extraction was carried out using a Quick DNA fungal/bacterial miniprep kit (catalogue number D6005) following the manufacturer's instructions.

Polymerase chain reaction

The genomic DNA was amplified using Polymerase Chain Reaction (PCR) to obtain a 260 base pair fragment of 28s rRNA. The PCR mix comprises of primer pairs (UI 5'-GTGAAATTGTTGAAAGGGAA-3' and U2 5'-GACTCCTTGGTCCGTGTT-3"), 0.25 ml Tag polymerase, and 2 uL of sample DNA. The amplification programme comprises of denaturation at 95oC for 45 s, annealing at 50 ^{o}C for 50 s, and extension at 72 ^{o}C for 5 min.

Statically analysis

Samples CFU/g were calculated using SPSS (version 24.0). DNA base sequences were compared with the GenBank databases of the NCBI using the BLAST program.

RESULTS

Prevalence of Fungal Isolates from Bambara Groundnut Samples in Different Locations in Enugu Metropolis

The results of the fungi isolates obtained from the 5 different points across the 3 markets are presented in Table 1. *A. niger* and *Trichothecium* spp were isolated from the New main market and Ogbete market. *A. flavus* was isolated across the three selected markets. *Colletotrichum lindemuthianum, Aspergillus fumigatus, Rhizopus stolonifera* and *Penicillium* spp were isolated from Ogbete in addition to *Trichothecium* spp. mentioned above. *Aspergillus ochraceus, Scolecosporae inflate* and *A. macrospora* were isolated only from Abakpa market. *Culvularia spp* was isolated from Abakpa and New main market.

Table 1. Occurrence of Fungal Isolates from Bambara Groundnut Samples in Different Locations in Enugu Metropolis

	Abakpa A					New Main Market					Ogbete				
Fungal isolate	A	B	C	D	E	G	H	I	J	K	L	M	N	O	P
Aspergillus niger	-	-	-	-	-	+	+	-	-	+	-	-	-	+	-
Aspergillus flavus	+	+	-	+	-	+	-	+	+	+	-	+	-	+	+
Aspergillus fumigatus	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-

Aspergillus ochraceus	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	
Curvularia spp	-	-	+	+	-	+	-	+	-	-	-	-	-	-	-	
Trichothecium spp	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	
Scolecosporae inflate	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	
Alternaria macrospara	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Colletotrichum lindemuthianum	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	
Penicillium spp	-	-	-	-	-	-	-	-	-	-	+	-	-	+	-	
Rhizopus stolonifer	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	

Key note: + = present; - = absent

Frequency (%) of prevalence of Fungal isolates on Bambara Groundnut from three Major Markets in Enugu Metropolis

The percentage frequency of prevalence in Table 2 shows that Bambara groundnut being sold in New Market has the highest mould isolate contributing 45% (35 out of 77 colonies) of the total isolates with location F having the highest mould isolates (16% of the figure). In the same view, total moulds isolated from the Abakpa market were investigated to follow the new market value, having 29% (22 out of 77) of the accurate value, while the Ogbete market was observed to have the lowest close figure (25%), indicating frequent stock replenishment.

Average Frequency (%) of prevalence of Fungi Isolated on Bambara Groundnut Samples in three Selected Markets in Enugu Metropolis

The average frequency (%) of the prevalence of the fungi isolated (Table 3) showed that *A. flavus* has 32% (highest) occurrence of the total isolates. It was closely followed by *Culvularia* (30%). Others in descending order are *A. niger* (12%), *A. fumigatus* (6%), *Penicillum spp* (5%), *Colletotrichum* spp (4%), *R. stolonifer* (3%), *Aspergillus ochaceus (3%)* and *Trichothecium* spp (3%), *A. macrospora* and *Scolecosporae* spp was 1% each as they were isolated only from Abakpa A and Abakpa D locations respectively.

Table 2. Average frequency (%) of prevalence of fungi isolated on Bambara groundnut samples in three selected markets in Enugu Metropolis

	Abakpa A					New Main Market					Ogbete					Total
Fungal isolate	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	
Aspergillus niger	-	-	-	-	-	4	1	-	-	2	-	-	-	2	-	
Aspergillus flavus	4	3	-	3	-	2	-	3	1	5	-	1	-	1	2	
Aspergillus fumigates	-	-	-	-	-	-	-	-	-	-	-	2	3	-	-	
Aspergillus ochraceous	1	-	-	-	1	-	-	-	-	-	-	-	-	-	-	
Curvularia sp	-	-	2	6	-	7	-	8	-	-	-	-	-	-	-	
Trichothecium sp	-	-	-	-	-	-	-	-	1	-	-	-	-	-	1	
Scolecosporae inflate	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	
Alternaria macrospara	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
Colletotrichum lindemuthian	-	-	-	-	-	-	-	-	-	-	1	-	2	-	-	
Penicillium sp	-	-	-	-	-	-	-	-	-	-	1	-	-	3	-	
Rhizopus stolonifer	-	-	-	-	-	-	1	-	-	-	1	-	-	-	-	

Occurrence	6	3	2	10	1	13	2	11	2	7	3	3	5	6	3	77
% occurrence	8	4	3	13	1	17	3	14	3	9	4	4	7	8	4	100

Key note: n = 5; - = absent

Table 3. Average frequency (%) of prevalence of fungi isolated on Bambara groundnut samples in three selected markets in Enugu Metropolis.

Fungal isolate	Number (%)
Aspergillus flavus	25.0 (32%)
Curvularia sp	23.0 (30%)
Aspergillus niger	9.0 (12%)
Aspergillus fumigatus	5.0 (6%)
Penicillium sp	4.0 (5%)
Colletotrichum lindemuthianum	3.0 (4%)
Trichothecium sp	2.0 (3%)
Rhizopus stolonifer	2.0 (3%)
Aspergillus ochraceous	2.0 (3%)
Scolecosporae inflate	1.0 (1%)
Alternaria macrospora	1.0 (1%)

Aflatoxin contamination load of the Bambara groundnut from three selected markets in Enugu Metropolis

The aflatoxin analysis as shown in Table 4 revealed that the Bambara groundnut samples produced aflatoxins. The sample from Obete markets (Ogbete N) produced the highest aflatoxin with concentration at 80 ppb, this is followed by new main market with concentration at 72 ppb (averaged) and then Abakpa market with concentration at 69 ppb (averaged). The presence of aflatoxin in the Bambara groundnut samples at concentrations higher than the WHO recommended limits for foods indicates that it is highly hazardous.

Molecular characterization of the aspergillus species isolated from the Bambara groundnut

From the preliminary analysis results obtained, 41 out of the total of 77 fungi isolated belong to *Aspergillus* species, 25 of which were identified morphologically as *A. flavus* were characterized using molecular techniques. DNA extraction results showed high molecular weight DNA (Figure 1) and the result for the amplification of the internal transcribed spacer region of the fungal isolates are shown in Figure 2.

Sequence alignment of the isolate sequences on the NCBI Genebank database identified 14 out of the 25 isolates as *A, flavus*, 6 isolates as other species of *Aspergillus* and 5 isolates as other genera of fungi (Table 5) with their unique accession numbers from the BLAST results of the isolate sequences and they were identified as *A. flavus* (Table 6). The *A. flavus* isolates characterized for Aflatoxigenicity showed that all 14 isolates were positive indicating the presence of the Aflatoxin regulatory gene in the isolates (Figure 3 and 4). In addition, the aflR gene for the detection of Aflatoxigenicity was amplified in them (Figure 5).

Table 4. Total Aflatoxin Concentration of the Samples from three Markets in Enugu Metropolis

Sample	Total Aflatoxin Concentration (µg/kg)
Abakpa A	70
Abakpa D	68
New Main Market F	69
New Main Market H	75
Ogbete N	80

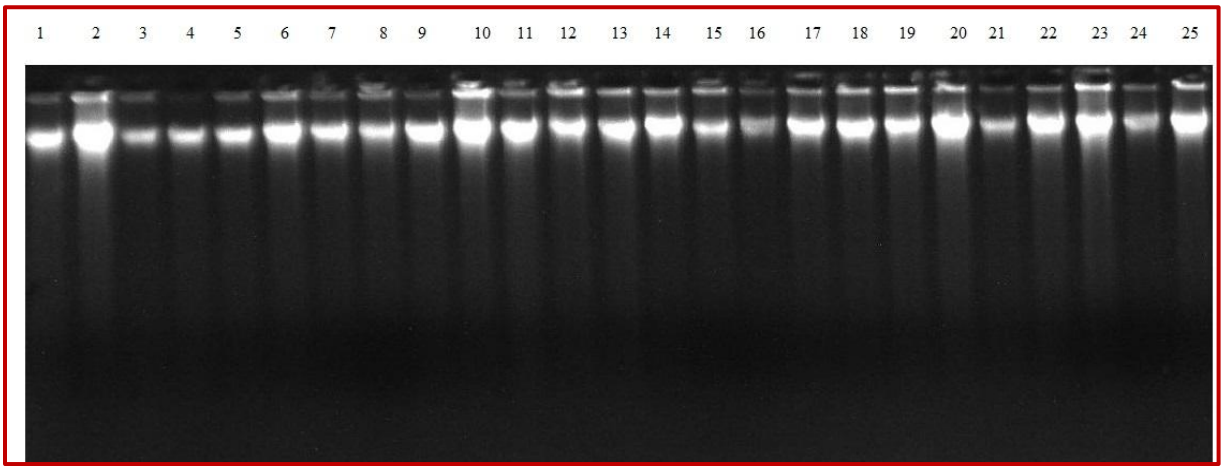

Figure 1. Agarose gel image of high molecular weight dna extracted from the Isolates

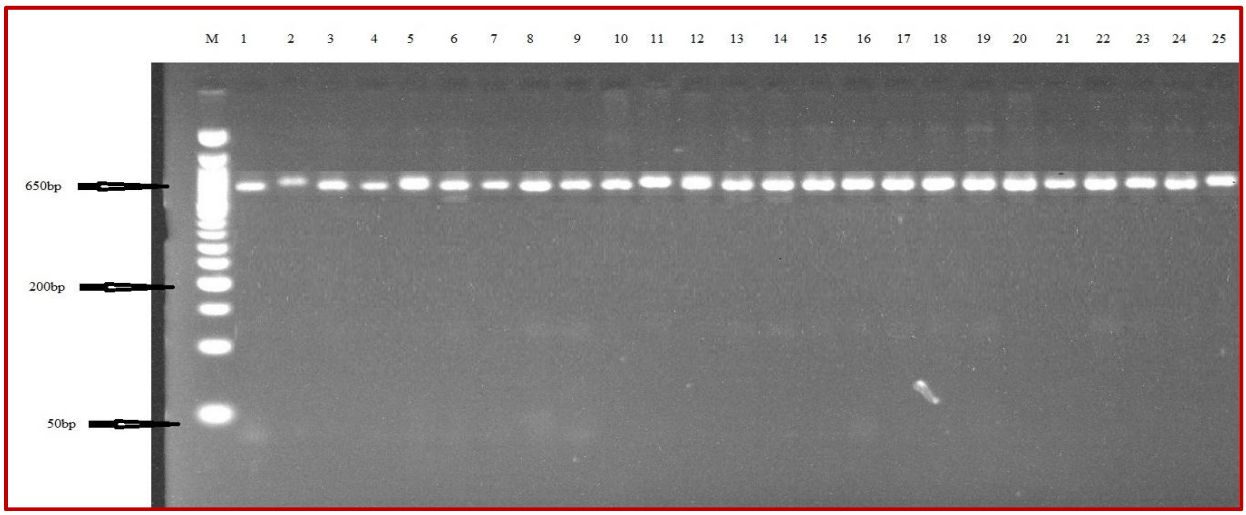

Figure 2. PCR Amplification of the Internal Transcribed Spacer (using ITS1 and ITS4 Primers) Region of the DNA Isolated at about 650pb.

Table 5. BLAST Results of Aligned Sequences of 25 Selected Isolates on NCBI

Isolate Code	Accession	Identified Organisms
1	MH511110	*Aspergillus flavus* strain L.A-1
2	KX098195	*Fungal sp.* strain Xmf236
3	MT139632	*Aspergillus sp.* strain 2F3F_AM
4	MG745384	*Aspergillus parvisclerotigenus* strain Maci262
5	MF668183	*Aspergillus parvisclerotigenus* strain CBS 121.62
6	MT446181	*Aspergillus flavus* strain ZMXL12
7	MT446181	*Aspergillus flavus* strain ZMXL12
8	MK226236	*Clavispora lusitaniae* strain D36

9	MT492458	*Aspergillus flavus* strain bpo4
10	MT594359	*Aspergillus flavus* strain 64-A1
11	MN565937	*Aspergillus flavus* strain omo2
12	KR611590	*Aspergillus flavus* strain PKM24
13	MK461562	*Aspergillus flavus isolate* strain BRM051244
14	MW011355	*Aspergillus aculeatinus* strain RCBBR_AEANW9
15	MG554264	*Alternaria sp.* strain GB-MG-3-3
16	MT669258	*Fungal sp.* strain BTf_8-1
17	MT446145	*Aspergillus sp.* strain ZMGL1
18	MG554231	*Aspergillus flavus* strain JN-YG-3-5
19	KP992924	*Penicillium indicum* strain zy24
20	MT594359	*Aspergillus flavus* strain 64-A1
21	MH863632	*Aspergillus alabamensis* strain CBS 125691
22	MH864265	*Aspergillus flavus* strain CBS 126856
23	KR611590	*Aspergillus flavus* strain PKM24
24	JX232269	*Aspergillus flavus* strain SGE22
25	MT072061	*Aspergillus flavus* strain QH06-08

Table 6. Unique accession numbers generated for the *Aspergillus flavus* isolates from the Bambara groundnut Samples

Isolate Code	Accession No	Identified Organism
1	OL336880	*Aspergillus flavus* strain BV1
6	OL336881	*Aspergillus flavus* strain BV2
7	OL336882	*Aspergillus flavus* strain BV3
9	OL336883	*Aspergillus flavus* strain BV4
10	OL336884	*Aspergillus flavus* strain BV5
11	OL336885	*Aspergillus flavus* strain BV6
12	OL336886	*Aspergillus flavus* strain BV7
13	OL336887	*Aspergillus flavus* strain BV8
18	OL336889	*Aspergillus flavus* strain BV10
20	OL336890	*Aspergillus flavus* strain BV11
22	OL336891	*Aspergillus flavus* strain BV12
23	OL336892	*Aspergillus flavus* strain BV13
24	OL336893	*Aspergillus flavus* strain BV14
25	OL336894	*Aspergillus flavus* strain BV15

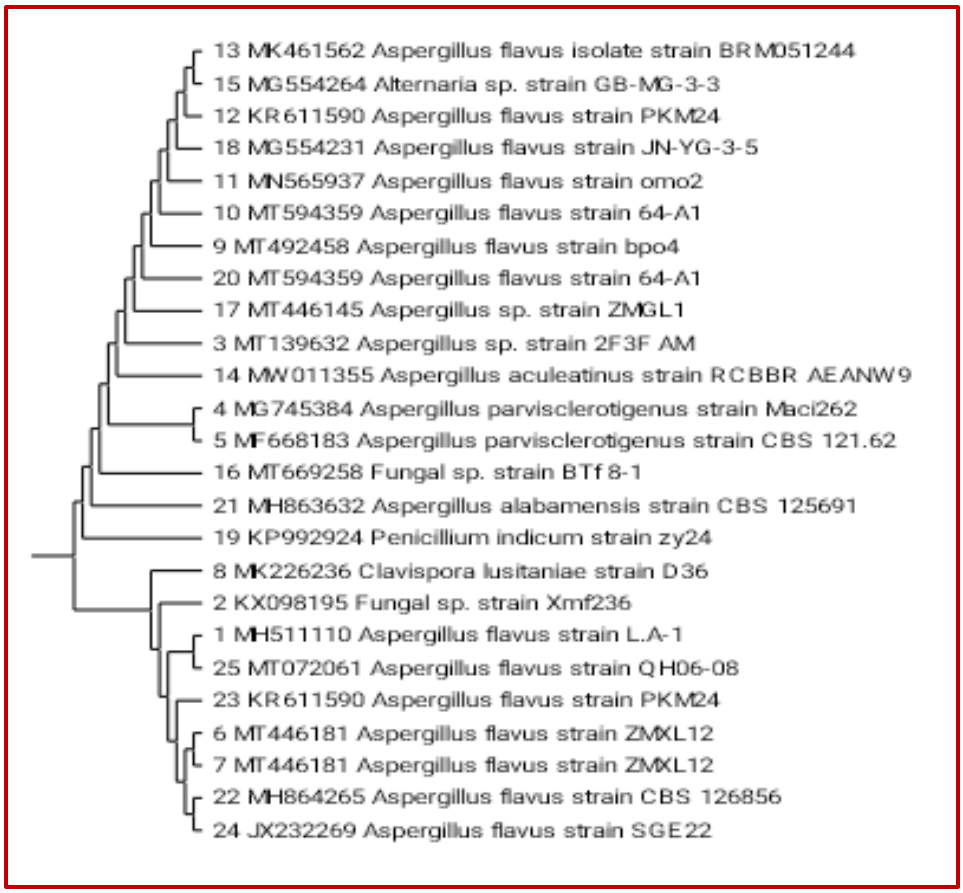

Figure 3. Phylogenetic relationship between the 25 isolates identified using molecular techniques

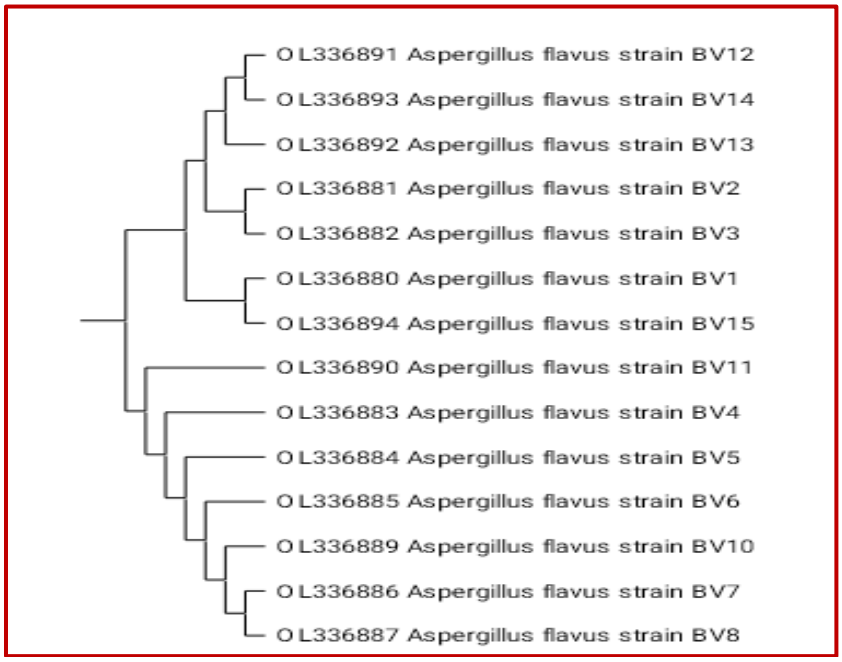

Figure 4. Phylogenetic relationship between the 14 isolates deposited on NCBI gene bank

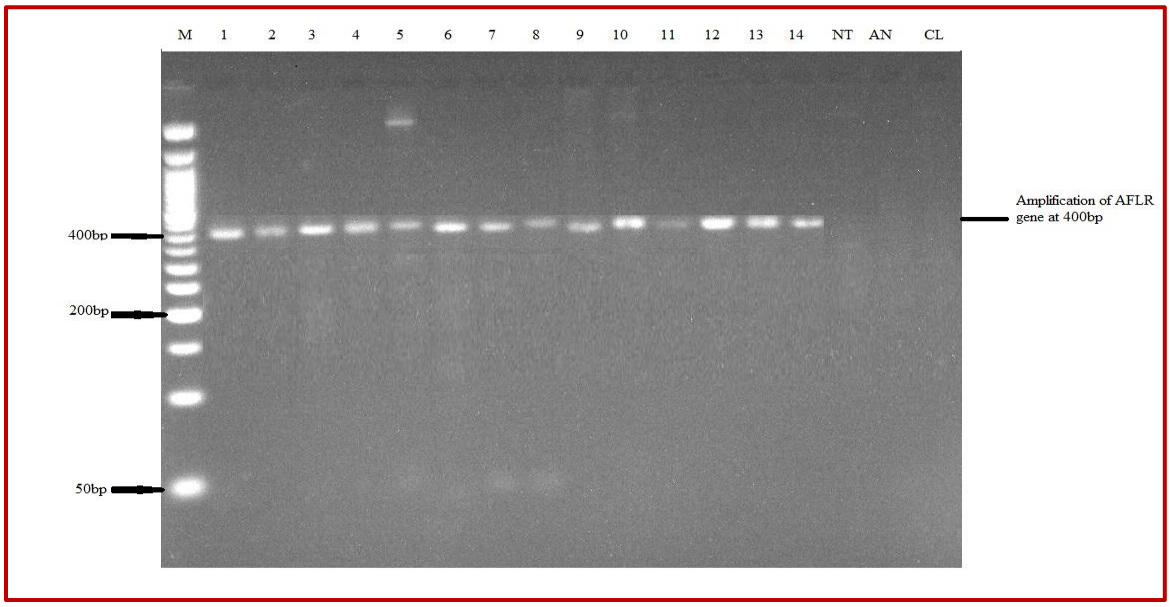

Figure 5. PCR amplification of the aflR gene of the isolated DNA. *Note:* Lane M is a 50bp ladder, lanes 1-14 are *A. flavus* isolates, lane NT is a No Template (control), lane AN is *A. niger* (control) and lane CL is *Curvularia* sp (control).

DISCUSSION

This study revealed the relationship between various species of fungi. The most dominant species among these fungal isolates as shown in my findings were *Aspergillus*. The occurrence of these fungal isolates might be due to the potentiality of fungal isolates to adapt to the prevailing conditions in the environment of the Bambara groundnut. This is in line with the report of Bamkefa et al. (2020), and Olagunju and Oluwatosin (2021) who stated that bambara nut provides a significant quantity of nutrients and enables the environment for fungal growth. A total of eleven fungi isolates from the Bambara groundnut was obtained, and four were different species of the genus *Aspergillus* (*niger, flavus, fumigatus* and *ochraceus*). One of the isolates consistent across the three markets, is *A. flavus*. *A. flavus* is a mould with the abundance of conidia. The high frequency of fungal isolates found corresponds to the report of Aroh (2018) who reported similar fungal isolates with frequencies from Bambara nuts sold at Nsukka Nigeria, and this may be as a result of the same geographical location and method of isolation. The majority of fungal species were found in greater abundance in new main market than in other locations, this is followed by Abakpa Market and then Ogbete market. *A. parasiticus* was not isolated from any of the Bambara groundnut samples which corresponds to the findings of Olagunju et al. (2021) who failed to isolate *A. parasiticus* from Bambara nut. He stated that this might be as a result of better sanitary practices in some parts of South Africa.

A. flavus found in this study is a known mould that produces afaltoxin and aflatoxin has been linked to human and animal health issues. Aflatoxins detected in this study could result from the use of infested BG seeds for planting, an infestation of the farmland before planting, insufficient post-processing handling practices, and storage of the seed in an unconducive environment. According to the findings of this study, all samples obtained from the market tested positive for aflatoxins, with overall aflatoxin contamination levels ranging from low to high, Abakpa (67 ppb), New main market (72 ppb) and Ogbete (80 ppb). The high level of aflatoxin contained in the Bambara groundnut samples found in this study corresponds to the report of Aroh (2018), and this may be a result of a low storage temperature of 25°C. It was discovered that low temperature favours the synthesis of aflatoxin.

The mucorales are responsible for the majority of human sickness. *Rhizopus* spp., *Aspergillus* spp., *Penicillium* spp. and *Altenaria* spp cause the majority of human sickness. Fungi belonging to the genera *Altenaria* is the most frequent mould that causes asthma and increases disease severity and mortality, they primarily induce allergies in adults who are susceptible to respiratory infections (Linn et al., 2022). It has been reported that 10 ppb AFB1

consumed regularly by rats can lead to fatal liver cancer, and aflatoxin in slightly higher concentrations of a few hundred parts per billion (ppb) — can cause problems for animals. In the United States, aflatoxin contamination of 20 ppb is allowed in feed grains and feeds; however, there is no tolerance for aflatoxin contamination in foods meant for human consumption (Sarma et al., 2017). Total aflatoxins (the sum of AFB1, AFB2, AFg1, and AFG2) have a guideline level of 20g/kg in food in the United States. For total aflatoxins in food, the European Community has a more restrictive ML of 4g/kg. Aflatoxin-free foods for infants are also required in Europe, as is an ML of 0.1g/kg for processed cereal-based foods and baby foods for newborns and young children (Vabi et al., 2018). Continuous exposure to aflatoxins-contaminated food and feed can cause immune system suppression in humans and animals, and infertility in men. As a result, attempts to export aflatoxins-contaminated crops have resulted in significant trade losses and diplomatic embarrassment for Nigeria (Tamura et al., 2021). In this study, nested PCR of the aflR gene which regulates the aflatoxin biosynthesis by controlling the expression of the nor-1 and ver-1 gene in *A. flavus*, gave a positive result for all 14 *A. flavus* isolates screened for aflatoxin production by amplification of the aflR gene at the expected base size of 400 bp. This result is in correlation with previous studies (Oluwatosin et al., 2020). In this research, it was discovered that the persistence of *A. flavus* in all three market samples might be due to high nutrient levels and thriving environment for these isolates.

CONCLUSION

This research work showed that the Bambara groundnut purchased from the three markets in Enugu Metropolis contains a high level of Aflatoxin-producing fungi. The most prevalent among these fungal isolates as shown by the results of this study were *A. flavus*, *Curvularia* spp and *A. niger*. Molecular Characterization of the fungal isolates, confirmed the presence of the Aflatoxin Regulatory Gene in the *A. flavus* screened using Molecular techniques. The consumption of Bambara groundnut contaminated by aflatoxin-producing fungi species poses major health risks as aflatoxin is harmful to human health due to its carcinogenic and teratogenic nature. Hence, there is a need to increase awareness about the potential risks involved in the use of Bambara groundnut as supplementary feeding for infants as well as adults. Farmers should be trained and educated on the use of bio-control products on farmland to drastically reduce the presence of pathogenic fungi on the farmland.

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AUTHORS CONTRIBUTION STATEMENT

I.V.C., B.B.A., F.O.A. Implementation: I.V.C., B.B.A., F.O.A. Data curation: I.V.C., O.C.O. Formal analysis: O.C.O. Writing original draft: I.V.C. Review-writing: I.V.C., B.B.A., O.C.O., F.O.A.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

ETHICS APPROVAL

Not applicable

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