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Aflatoxin and molecular characterization of isolates from stored food in some selected Local Government in Ekiti State

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Abstract

Aflatoxins are secondary metabolites produced by fungi species from the genus Aspergillus, notably *A. flavus*, *A. parasiticus* and *A. nomius*, which develop naturally in food products and tend to cause wide array of toxic effects in several animal species, including humans. Aflatoxins are typically reported in dry food commodities such as cereals, spices, and dry fruits. This research was conducted in Ido-Osi, Efon, and Emure Local Government Areas in Ekiti State, Nigeria. Three samples; Local rice (*Oryza sativa* L), Maize (*Zea mays*) and Groundnut (*Arachis hypogaea* L) were obtained from three popular markets from each local government. About 10 grains of each sample were selected at random into separate petri plates and surface sterilized in 1% hypochlorite for 3 minutes. The grains were rinsed several times with distilled water. The organisms associated with the samples were isolated using Direct plating method while the aflatoxin content was determined using ELIZA. The result showed the occurrence of five (5) different fungal species distributed among the stored foods. These include: *Aspergillus spp.*, *Rhizopus spp*. *Penicillium spp. Mucor spp. and Botrytis spp*. The species determination was confirmed by PCR with the primers specific for the ITS-rDNA region sequencing. The organisms were confirmed to the species level to be; *Aspergillus flavus* and *Trichoderma viridie.* Aflatoxin B1 was present in all the samples in which sample 6 has the highest values of 240ppb while aflatoxin B2 was relatively low. The prevalence of *Aspergillus flavus* in the stored foods is above 50%, which is very high and indicate the possible highlevel production of aflatoxin in food samples under study.

Keywords: Isolation; Characterization; Isolates; Stored foods

1. Introduction

Aflatoxins are secondary metabolites produced by fungi species from the genus *Aspergillus*, notably *A. flavus*, *A. parasiticus* and *A. nomius*, which develop naturally in food products and cause a wide array of toxic effects in several animal species, including humans [1]. Aflatoxins are typically reported in dry food commodities (cereals, spices, nuts and dry fruits), while the metabolic products of aflatoxins, such as AFM1 and AFM2, are reported in milk [2, 3]. Aflatoxin is a mycotoxin produced by two types of *Aspergillus* which are *Aspergillus flavus* and *Aspergillus parasiticus*. Mycotoxin produced by *A. flavus* is most often found when certain grains such as wheat, corn, beans, and rice are grown under stressful conditions such as high moisture and temperature, but it can also be found on harvested grains if the grains are stored damp [4]. Aflatoxin can also be found in milk, eggs, and meat products when ruminants feed on contaminated foods [5]. It was first recorded in 1960 in the United Kingdom when 100,000 turkeys and other poultry died in a single event. The cause of this was the birds fed on groundnut meal contaminated with aflatoxin B1 [6]. The occurrence of aflatoxins in food appears to be very important for health and economic reasons

There are different types of aflatoxin mycotoxins. *A. flavus* produce only aflatoxins B1 and B2, whereas *A. parasiticus* produce aflatoxins B1, B2, G1, and G2. Due to its carcinogenic effect, contamination of grains, milk and dairy products

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with aflatoxin may pose a risk for consumers. Aflatoxins are byproducts from the fungi *Aspergillus flavus* and the name Aflatoxin was given by the virtue of its origin (*A. flavus* - *afla*). Not only *A. flavus*, but other strains of Aspergillus like *Aspergillus parasiticus, Aspergillus nomius, Aspergillus pseudotamarii, Aspergillus bombycis, Aspergillus toxicarius* and *Aspergillus parvisclerotigenus* also produce Aflatoxin [7].

[8] isolated *Aspergillus flavus, Aspergillus niger, Aspergillus fumigatus, Rhizopus stolonifer, Mucor mucedo* and *Alternaria* spp in decreasing order from fruits in Utako market, Abuja, Nigeria. Similarly, Al-Hindi *et al.* [9] showed that the fungal genera *Aspergillus, Penicillium, Fusarium* and *Rhizopus* were isolated from agar wood where *Aspergillus flavus, Aspergillus parasiticus* and *Aspergillus niger* were the dominant fungi.

Luttfullah and Hussain [10] also reported that the fungal genera isolated from banana and mango were *Aspergillus* sp, *Fusarium* sp and *Cladosporium* sp while pineapple showed negative result with no occurrence of fungi isolated.

Similarly, Jedidi *et al.* [11] reported that the dominant fungal genera isolated and identified from maize and wheat in Tunisia were *Alternaria, Eurotium, Aspergillus* and *Penicillium* in decreasing order while Ennouari *et al.* [12] also reported that the fungal genera isolated and identified from durum wheat were *Alternaria, Fusarium* spp and *Aspergillus* spp in decreasing order.

The aim and objective of this study is to isolate fungi from stored rice, maize and groundnut, characterize and classify the fungi isolated from the samples.

2. Material and methods

Samples of local rice (*Oryza sativa* L), Maize (*Zea mays*) and Groundnut (*Arachis hypogaea* L) were bought from three popular markets *namely* Idi- Osi-(Ifaki, Ido and Ora market), Efon-(within the town) and Emure-(within the town). The samples from various markets were conveyed to the Laboratory in separate clean polythene bag.

2.1. Sample Preparation

The foreign particles present in the grains were carefully removed by picking with hand. For each sample, ten grains were randomly selected into separate petri dishes. The grains were surface sterilized in 1% hypochlorite for 3 minutes. The grains were further rinsed several times with distilled water.

2.2. Isolation of Fungi

Each sample was plated on different PDA plates. A sterile forceps was used to aseptically inoculate the samples into the PDA plates. The plates were incubated at 25 °C for 5 days. The fungal cultures were sub cultured until pure colonies were obtained by successive hypha tip transfer.

2.3. Identification of Fungi isolates

The fungal isolates were identified using cultural and morphological features such as colony growth pattern, conidial morphology and pigmentation. All the colonies were observed. The isolates were examined under bright daylight for the colour of the culture. The fungal culture were viewed under the light microscope with ×10 and ×40 objective lens. The morphological characteristics and appearance of the fungal organisms seen were compared in accordance with Adebayo-Tayo *et al.* [13], Onuorah *et al.* [14], Klich [15] and Samson and Varga [16]

2.4. Extraction of aflatoxins from samples

The sample (maize and rice) were grinded with romer mill, and thoroughly mixed. 20g of ground samples were weighed for extraction, and blended with 100 ml of 70% methanol for three minutes using waring blender. The blended mixture was poured into a 250 ml Pyrex conical flask and seal flask with paraffin. The sample was shaken using orbit shaker at 4 x 100 rpm for 30 minutes. The mixed blend was filtered with No 1 quantitative Whatman filter paper, 185 mm into a clean conical flask rinsed with methanol.

The filtrate (40 ml) was poured into a separating flask, and 20 ml of distilled water was added. 25 ml of dichloromethane was also added. It was gently shaken and left separating flask to stand (allowing the mixture to separate into top and bottom phases). The extract was drained through a bed of 20 g anhydrous sodium sulphate into a 150 ml white plastic beaker. 10 ml of dichloromethane was added to the remaining mixture in the separating flask, and gently shaken to

allow separation. The extract was drained through a bed of 20g anhydrous sodium sulphate into a 150 ml white plastic beaker (that contains the first extract), and the extract was allowed to dry overnight in the fume hood.

2.5. Quantitative assay of extracts

The developed plates were viewed under the ultraviolet light- box (wavelength = 365 nm) to see whether each extract fluoresces or not. Those with fluorescence and those without are compared with the standards.

Quantitatively, extracts showing fluorescence during qualitative analysis are further subjected to quantitative analysis to ascertain total amount aflatoxins (B1, B2, G1 and G2) in the sample. This is done with the aid of CAMAG TLC scanner 3; which enables quantitative evaluation of densitometry data to be generated.

2.6. Molecular Characterization of Fungal Isolates

2.6.1. Fungal DNA Extraction and Preparation

Fungal cells, 50-100mg (Net weight) which have been resuspended in up to 200ul of water or up to 200mg of tissue was added to a ZR Bashing Lysis Tube. 750ul Lysis Solution was added to the tube. It was secured in a bead fitted with 2ml tube holder assembly and processed at maximum speed for 5 minutes or more. The ZR Bashing Bead Lysis Tube was centrifuged in a micro centrifuge at above 10000 x g for 1 minute. 400ul supernatant was transferred to a Zymo-Spin IV Spin Filter (orange top) in a collection tube and centrifuged at 7000 x g for 1 minute. The base of the Zymo-Spin IV Spin Filter was snapped off prior to use. 1200ul of fungal DNA Binding Buffer was added to the filtrate in the collection tube. 800ul of the mixture was transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10000 x g for 1 minute. The Zygo-Spin IIC column has a maximum capacity of 800ul. The flow through from the collection tube was discarded. The mixture was transferred back to the Zymo-Spin IIC column in a collection tube and centrifuged at 10000 x g for 1 minute. 200ul DNA pre-wash Buffer was added to the Zymo-Spin IIC column in the new collection tube and centrifuged at 10000 x g for 1 minute. 500ul fungal DNA wash Buffer as added to the Zymo-Spin IIC column and centrifuged at 10000 x g for 1 minute. The Zygo-Spin IIC column was transferred to a clean 1.5ml micro centrifuge tube and 100ul (35ul minimum) DNA Elution Buffer was directly added to the column matrix and centrifuged at 10000 x g for 30 seconds to elite the DNA.

2.6.2. Polymerase Chain Reaction (PCR)

PCR cocktail mix consists of 2.5ul of 10x PCR buffer, 1ul of 25mM $MgCl_2$, 1ul of DMSO, 2ul of 2.5 mMDNTPs, 0.1ul of 5u/ul Taq DNA polymeease and 3ul of 10ng/ul DNA.

Initial denaturation at 94°C for 5 minutes, followed by 36 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds and elongation at 72°C for 45 seconds followed by a final elongation step at 72°C for 7 minutes and hold temperature at 10°C forever. PCR amplification was carried out in GeneAmp PCR 9700. Amplified fragments were visualized on safe view-stained 1.5% Agarose electrophoresis gels. The size of the amplicon was about 600pb. DNA ladder used was Hyper Ladder from Bioline.

2.6.3. PCR Fragments Purification

The Centricon-100 column was assembled according to the manufacturer's recommendation. 2ml of deionized water was added to the column. The entire sample was added to the column. The column was spun at 3000 x g in a fixed-angle centrifuge for 10 minutes. The waste receptacle was removed and the collection vial was attached. The column was inverted and spun at 270 x g for 2 minutes to collect the sample. This should yield approximately 40-60ul of sample. Deionized water was added to bring the purified PCR fragments to the original volume.

2.6.4. Fungal DNA Sequencing (Forward Reaction)

Sanger sequencing was performed using 3130 XL Genetic Analyzer from Applied Biosystems. The sequence of primer used are

ITS4: TCCTCCGCTTATTGATATGC and ITS5: GGAAGTAAAAGTCGTAAGAAGG. The sequence alignment and required editing if he obtained sequence was carried out using the Bio Edit software. The obtained DNA sequence was blasted on the NCBI Gene library for finding similar sequences. Only those sequences were included that showed highest sequence similarities with the query sequence.

3. Results

Table 1 Morphological characteristics of fungal isolates from stored foods in Ekiti

Samples	Morphological characteristics	Fungi identified
R1a	Yellow green colour on the surface, cream colour on the reverse, woolly in texture, septate hyphae	*Aspergillus flavus*
R1b	Black colour on the surface, yellow colour on the reverse, velvety in texture, filamentous, septate hyphae	*Aspergillus niger*
R1c	White to grey colour on the surface, pale white colour on the reverse, filamentous, cottony in texture, non-septate hyphae	*Rhizopus* spp
R1d	White to brown colour with dark spots on the surface, dark coloured reverse, filamentous, woolly in texture, septate hyphae	*Botrytis* sp
R1e	Dark green colour on the surface, bright orange yellow colour on the reverse, suede like surface, velvety in texture, filamentous, septate hyphae	*A. parasiticus*
GN1a	Green with white colour on the surface, orange coloured wrinkled reverse, filamentous, powdery in texture, septate hyphae	*Penicillium* sp
GN1b	Greenish colour on the surface, pale yellow colour on the reverse, powdery in texture, filamentous, septate hyphae	*Aspergillus* sp.
GN1c	White to grey colour on the surface, pale white colour on the reverse, filamentous, cottony in texture, non septate hyphae	*Rhizopus* sp
GN1d	White to grey brown colour on the surface, white coloured wrinkled reverse, fluffy to cottony in texture, non septate hyphae	*Mucor* sp
M1a	Yellow green colour on the surface, cream colour on the reverse, woolly in texture, septate hyphae	*Aspergillus flavus*
M1b	Green with white colour on the surface, orange coloured wrinkled reverse, filamentous, powdery in texture, septate hyphae	*Penicillium* sp
M1c	White to brown colour with dark spots on the surface, dark coloured reverse, filamentous, woolly in texture, septate hyphae	*Botrytis* sp
M1d	White to grey colour on the surface, pale white colour on the reverse, filamentous, cottony in texture, non septate hyphae	*Rhizopus* sp
M1e	White to grey brown colour on the surface, white coloured wrinkled reverse, fluffy to cottony in texture, non septate hyphae	*Mucor* sp
M1f	Greenish colour on the surface, pale yellow colour on the reverse, powdery in texture, filamentous, septate hyphae	*Aspergillus* sp.

R: Rice; GN: Groundnut; M: Maize.

Table 2 Molecular characterization of the isolates

Isolate	Strain	E-value	ITS4rDNA identity (%)	Isolates' Identity
B1	*Aspergillus flavusT13*	0.0	90.73	*Aspergillus flavus*
B2	*Aspergillus flavus* Egy3	0.0	89.56	*Aspergillus flavus*
B3	*Trichoderma viridie*	0.0	80.19	*Trichoderma viridie*
B4	*Aspergillus flavus*	0.0	90.91	*Aspergillus flavus*

Table 3 Aflatoxin content of the suspected samples

Sample code	Aflatoxin concentrations (ppb)bc			
	B1	B2	G1	G2
1	23	6	0	0
2	33	15	0	0
3	38	6	0	0
4	25	6	0	0
5	36	7	0	0
6	240	15	0	0

4. Discussion

Rice and maize are the second and third respectively, the most important cereal grains worldwide after wheat. Rice is directly consumed by humans. Groundnut is a staple food eaten in the world especially in Africa. Groundnut and maize are consumed directly by humans directly humans and indirectly by animals. In Nigeria, these foods are eaten throughout the year and as such the need for storage.

The number of isolate *Aspergillus* spp was the highest isolate ten (10) followed by *Rhizopus* sp and *Penicillium* sp. The isolates were *Aspergillus flavus*, *Aspergillus parasiticus*, *Aspergillus* niger, Aspergillus sp, *Rhizopus* sp, *Penicillium* sp, *Mucor* sp and *Botrytis* sp. In all analysed samples, the prevalent fungal genera was *Aspergillus* which could have been as a result of their ubiquity. This finding is similar to that of Nyirahakizimana *et al.* [17] who reported that the genus *Aspergillus* was the major genus that affects nuts and seeds. Similarly, this is in agreement with the result of Kenjo *et al.* [18] who reported that *Aspergillus flavus and Aspergillus niger* were the most visible and frequent fungi in Almonds from Japan.

Fungal species isolated from rice were *Aspergillus flavus, Aspergillus parasiticus, Aspergillus niger, Botrytis* sp. The result of this study is in agreement with the work of Taligoola *et al.* [19] who reported that *Aspergillus flavus, Aspergillus parasiticus, Aspergillus niger* were isolated from rice form Libya. Similarly, this is similar to those of Amanloo *et al.* [20] who reported that *Aspergillus flavus, Aspergillus parasiticus, Aspergillus niger, Aspergillus fumigatus,* and *Rhizopous* sp were isolated from rice from Iran.

Fungal genera isolated from groundnut were *Penicillium* sp, *Rhizoous* sp, *Mucor* sp and *Aspergillus* sp. This is similar to the findings of Adebesin *et al*. [21] who identified *Aspergillus flavus, Aspergillus niger, Aspergillus tamanii, Penicillium citrinum, Rhizopus stolonifer* in roasted groundnut sold in Bauchi State of Nigeria. Similarly, this is in agreement with the study of [22] who observed *Mucor* spp, *Aspergillus* spp, *Penicillium* spp, *Curvularia* spp, *Fusarium* spp and *Rhizopus* spp in groundnut seeds sold in Ailero Central Market. Also, this is consistent with those of the study of Akinnibosun and Osawaru [23] who found that *Neurospora* spp, Trichoderma spp, *Aspergillus niger, Aspergillus flavus, Mucor* spp, *Rhizopus* spp, *Penicillium* spp and *Fusarium* spp in peeled and unpeeled groundnut sold in Benin.

Fungal genera isolated from maize were *Mucor* sp, *Aspergillus* sp, *Botrytis* sp, *Rhizopus stolonifer* sp, *Penicillium* sp, and *Aspergillus flavus.* This is in agreement with the result of Samuel *et al.* [24] and Muthomi *et al.* [25] who reported that maize samples had a higher contamination by *Aspergillus* spp. The reason for the high contamination by *Aspergillus* spp is because of their ability to colonize substrates [26]. This is also similar to the finding of Njobeh *et al.* [27] who reported that *Penicillium* sp and *Rhizopus* sp were isolated from maize in Cameroon. Similarly, this is in agreement with the result of the study of Rosemary *et al.* [28] who reported that the fungal species *Penicillium* sp, *Rhizopus* sp, *Aspergillus* sp and *Mucor* sp were isolated in maize from Enugu State of Nigeria.

The identity of the isolates which was determined by molecular characterization shows that they hire similarities to strains of isolates in the *Aspergillus* and Trichoderma genus except for two that did not show any result. The species determination was confirmed by PCR with the primers specific for the ITS-rDNA region sequencing. Out of the 4 isolates (B1, B2, B3, and B4), B1, B2, and B4 were *Aspergillus flavus,* B3 was *Trichoderma viridie.* Prevalence of one dominant specie and three different strains of *Aspergillus* were shown. The species was *Aspergillus flavus* and three strains of *Aspergillus flavus.* The prevalence of *Aspergillus flavus* in the stored foods is above 50%, which is very high, and indicates

the possible high level production of aflatoxin in the food samples under study. This study has shown that various storage fungi are associated with the grains (maize, groundnut and rice) and previous studies have shown that toxigenic fungi are associated with food and feed commodities. These fungi proliferate during storage. The presence of these fungal species is a reason for concern because most of the species isolated are producer of mycotoxins such as Aflatoxin, Patulin, Ochratoxin A and mycotoxin contamination is a serious food safety issue worldwide. They are known to cause numerous effects on exposed humans and animals [29].

5. Conclusion

So, judging from the high incidence of fungal contamination observed in these grains, it is evident that consumers might be at risk of chronic mycotoxin exposure since these fungal species are known to produce toxins. Hence, the prevalence of fungal of fungal contamination in the samples, warrant intervention strategies to minimize their occurrence.

In view of the fungal contamination detected from the market samples, proper drying, storage and packaging of these grains in air tight containers to reduce settling of droplets and spores should be done.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest.

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