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RESEARCH ARTICLE

MOLECULAR CHARACTERIZATION OF MYCOTOXIN PRODUCING MOULDS ISOLATED FROM STORED PRODUCTS OF GRAINS (RICE, MAIZE, WHEAT AND GROUNDNUT)

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ABSTRACT

Stored products of grains studied were rice, maize, wheat and groundnuts. These cereals and legume are among the staple food sources for people living in developing countries. 160 samples of the stored products of grains were randomly collected from different markets. They were stored for a period of 2-4 months in different packaging materials and analysed for the presence of mycotoxigenic moulds and production of mycotoxins. Standard microbiological and molecular methods were used in the isolation and identification of moulds. A multimycotoxin method based on Liquid Chromatography tandem mass spectrometry was applied to investigate both the qualitative and quantitative occurrence of mycotoxins. Mycotoigenic moulds species identified using 18S rRNA sequences were *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus niger*, *Aspergillus brunneoviolaceus*, and *Penicillium chrysogenum.* Their percentage occurrence were *Aspergillus flavus* (46%) followed by *Aspergillus tamarii* (23%), *Aspergillus niger* (18%), and *Penicillium chrysogenum* (9%) while the least was *Aspergillus brunneoviolaceus* (4%). The mycotoxins detected were Aflatoxin B_1, Aflatoxin B_2, Aflatoxin G_1, Aflatoxin G_2, Ochratoxin A, Citrinin, Dihydrocitrinone, Fumonisin B_1, Fumonisin B_2, Fumonisin B_3, Fumonisin B_4, Zearalenone, Deoxynivalenol and Nivalenol. The largest concentration of mycotoxins detected from stored products of grains were fumonisin (1350 ± 10.000 µg/kg), followed by aflatoxins (1265.3 ± 1.327 µg/kg), then Citrinin (Dihydrocitrrinone) (709.8 ± 1.039 µg/kg), Trichothecenes: (Nivalenone Deoxynivalenone) (642.2 ± 1.900 µg/kg), Ochratoxin A (371.8 ± 1.616 µg/kg), and the least being Zearalenone (358.5 ± 2.500 µg/kg). Rice (1286.3 ± 29.689 µg/kg) contained the largest amount of the various mycotoxins, followed by wheat (1166.8 ± 0.901 µg/kg), and then groundnuts (1142.9 ± 10.488 µg/kg) while maize (1111.6 ± 9.810 µg/kg) had the least quantity of mycotoxins. The stored products of grains were mainly contaminated with *Aspergillus* species and contained different mycotoxins found to be of public health importance. The need for proper harvest and storage of grains cannot be overemphasized.

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INTRODUCTION

In developing countries, Rice (*Oryza sativa*) Maize *(Zea mays*) Wheat (*Triticum aestivum*) and Groundnut (*Arachis hypogaea*) are essential food crops. They are good sources of nourishment for the body (1). Mycotoxigenic moulds are usually of the genera *Aspergillus*, *Penicillium* and *Fusarium* (2). The negative effects caused by stored moulds include damage to grains, change in the organoleptic quality of grains, deficiency in nutrient, difficulty in germination, mycotoxins development (3)(4). Mycotoxins are known as toxic secondary metabolites, which are mainly produced by moulds that usually invade foods before and after harvest and also during storage.

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There are about four hundred mycotoxins that have been recognized in the world. Mycotoxins that have public significance include the aflatoxins, fumonisins, zearalenone, ochratoxins, trichothecenes (mainly dexoynivalenol and T-2), patulin and citrinin (5). The continuous contamination and exposure to mycotoxins in foods on a regular basis usually leads to a wide range of health complications. Aflatoxins B_1 and Fumonisins have been established to cause hepatocarcinoma, oesphageal cancer and deaths in humans (5). Drying quickly and evenly remains the best way of preventing mycotoxigenic moulds that produce mycotoxins (6).

MATERIALS AND METHODS

SAMPLE COLLECTION AND PREPARATION: A total of one hundred and sixty (160) Whole grains /fine powder of rice, maize, wheat and groundnut randomly obtained from the markets were stored in four different storage materials (sack,

polyethene, plastic containers and metal containers) for two to four months at ambient temperature in a dry environment. Thirty grams (30 g) each of the unstored and stored samples were labeled and transported immediately to laboratory and kept in cool place prior to mycological analysis (7)

ISOLATION OF FUNGI: Three mycological media (Malt Extract Agar, Potato Dextrose Agar and Sabouraud Dextrose Agar) were prepared according to standard methods. An antibacterial agent (50 mg/l, chloramphenicol) and 0.1ml of lactic acid were incorporated to inhibited the bacterial and yeasts growth respectively (8). Standard dilution and streaking technique method was adopted. The samples were serially diluted up to dilution factor of 10^{-3} and 10^{-5}. One-tenth milliliter (0.1ml) of suspension was inoculated onto the freshly prepared surface dried media and incubated at 25 ± 2°C for 7 days for mould growth. Moulds grown on media were subculture on various media for further characterization and identification (9).

MORPHOLOGICAL AND MICROSCOPIC IDENTIFICATION

The isolated moulds were identified based on colonial morphology and microscopic examination. The moulds were mounted on a clean grease slide, flooded with lactophenol-cotton blue stain to determine mould structures. Microscopically, moulds were identified on the basis of spore characteristics, pigmentation and septation (10).

MOLECULAR CHARACTERIZATION OF MOULDS

The DNA of mould isolates were extracted using deoxyribonucleic acid extraction protocol as described by (11).

The extracted DNA was amplified using polymerase chain reaction (PCR) amplification protocol described by (11).

SEQUENCING PROTOCOL: PCR products were cleaned using Exosap Protocol, sequenced using the Nimagen Brilliant dye Terminator cycle sequencing kit (12). The sequenced data were subjected to Basic Local Alignment Search Tool Nucleotide (BLASTn) to identify corresponding organisms from National center for bioinformatics information (NCBI) as described by (13).

PHYLOGENETIC TREE ANALYSIS: The obtained nucleotide sequence was analyzed using software, the geneious software version 4.0 (14).

LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

A multimycotoxin method based on Liquid Chromatography Tandem Mass Spectrometry was applied to investigate the occurrence of different mycotoxins. Samples of rice, maize, wheat and groundnut were analyzed. Samples were homogenized and kept in slant glass bottle and stored at 2-8°C for further analysis (qualitative and quantitative analysis of mycotoxins).

SAMPLE PREPARATION AND LC-MS/MS DETERMINATION: To 5 g of each sample, 20 ml of extraction solvent (acetonitrile/water/acetic acid 79: 20: 1, v/v/v) were added together. Extraction, dilution, and analysis, detection and quantification were performed as described by (15).

RESULTS AND DISCUSSION

The various moulds that were isolated from the unstored and stored products of grains were characterized morphologically and microscopically. They were further identified by sequencing of 18S rRNA gene using ITS1 and ITS4 primers. All samples were contaminated with different moulds. Table 1 shows the species of moulds, *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus niger*, *Aspergillus brunneoviolaceus*, and *Penicillium chrysogenum* isolated from the stored and unstored products of grains.

Table 1. Cultural and microscopic characteristic of identified isolates

Cultural	Microscopic	Probable organism
Colonies are greenish in colour	Hyphae are septate and hyaline.	*Aspergillus* sp
Rusty brown or dark brown	Conidia head with long chain of conida	*Aspergillus* sp
Black in colour	Septated hyphae, long smooth and colourless	*Aspergillus* sp
Brown to dark brown	Hyaline or pigmented longer stipes	*Aspergillus* sp
Blue green with a yellowish pigment	Septate hyphae branched	*Penicillium* sp

Table 2. Sequence identity of various moulds

S/N	Sample	Seqquence id	Percentage (%)	Ncbi match	Isolate
1	2	NR111041.1	99	*Aspergillus flavus* NR135325	*Aspergillus flavus*
2	11	NR138279.1	97	*Aspergillus brunneoviolaceus* NR138279	*Aspergillus brunneoviolaceus*
3	17	AY373852.1	91	*Aspergillus niger* AY373852	*Aspergillus niger*
4	22b	NR138306.1	99	*Penicillium chrysogenum* MH793845	*Penicillium chrysogenum*
5	23	AF004929.1	100	*Aspergillus tamarii* MN339986	*Aspergillus tamarii*

MOLECULAR IDENTIFICATION OF ISOLATED MOULDS: Five moulds were identified by the Genomic DNA extraction, amplification and sequencing. The sequenced data were subjected to Basic Local Alignment Search Tool Nucleotide (BLASTn) to identify corresponding organisms from National center for bioinformatics information. The stored products of grains (rice, maize, wheat and Groundnut) analyzed had different types of moulds as shown in (Table 1). The identified moulds were *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus niger*, *Aspergillus brunneoviolaceus*, and *Penicillium chrysogenum.* These results were similar to the work of (16).The most frequent genus isolated was *Aspergillus* with four different species namely *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus niger*, *Aspergillus brunneoviolaceus*. Result in Table 2 showed that the predominant mould species were in the order *Aspergillus flavus* (42%), *Aspergillus tamarii* (22%), *Aspergillus niger* (21%), and *Penicillium chrysogenum* (10%) while the least was (5%) *Aspergillus brunneoviolaceus*. *Aspergillus flavus* produce Aflatoxins and *Aspergillus* produce Ochratoxin A and

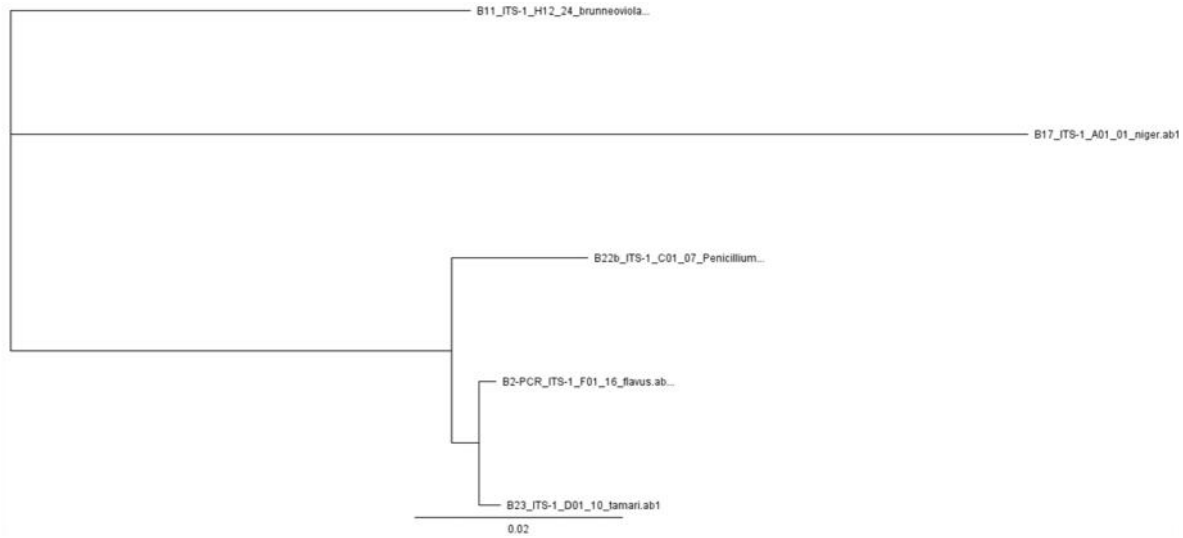

Figure 1. The Phylogenic tree constructed using the geneious software version 4.0 [12]

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4	22b	NR138306.1	99	*Penicillium chrysogenum* MH793845	*Penicillium chrysogenum*
5	23	AF004929.1	100	*Aspergillus tamarii* MN339986	*Aspergillus tamarii*

Table 3. Frequency and Percentage Occurrence Of Mycotoxigenic Moulds.

Moulds	Rice Frequency	Percentage	Maize Frequency	Percentage	Wheat Frequency	Percentage	Groundnut. Frequency	Percentage	Total Frequency	Total Percentage
A .flavus	11	34%	50	60%	8	27%	30	32%	99	42%
A. tamarii	7	22%	20	24%	1	3%	25	28%	53	22%
A .niger	3	9%	8	10%	18	60%	23	25%	52	21%
A.brunneoviolaceus	5	16%	3	3%	1	3%	2	2%	11	5%
P.chrysogenum	6	19%	3	3%	2	7%	12	13%	23	10%
	32	100%	84	100%	30	100%	92	100%	238	100%
	14%		35%		12%		39%			

Table 4. Qualitative And Quantitative Analysis Of Mycotoxins.

LOQ	0.722389402	0.211326816	0.54253691	1.69	1.52	2.45	3.76	7.9842379	7.08101209	19.23567874	7.0810120	0.633360953	9	2.565522952
LOD	0.2167168	0.063398045	0.16276107	0.51	0.46	0.73	1.13	2.395271379	2.124303627	5.770703622	2.124303627	0.19000828	3	0.769656885

	Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2	Ochratoxin A	Citrinin	Dihydrocitrinone	Fumonisin B_1	Fumonisin B_2	Fumonisin B_3	Fumonisin B_4	Zearalenone	Deoxynivalenol	Nivalenol
Maize	58.2	60.8	84.7	67.8	87.8	27.1	147.3	85	85	85	85	85	80	72.9
Wheat	84.3	86.1	79.5	80.5	96.9	43.9	115.5	85	85	85	85	100.1	80	60
Rice	97.4	96.3	97.5	94.2	100.5	80	100.1	85	85	85	85	99.2	80	101.1
Groundnut	81.5.	69	65.3	62.2	86.6	98.8	97.1	85	85	85	85	74.2	80	88.2

their presence in stored and unstored food products can be detrimental to human health (17). Table 2, result showed the frequency and percentage occurrence of moulds from different grains, with *A. flavus* (60%) from maize and *A. niger* from wheat (60%) predominating. This finding was similar to those reported by (18). The occurrence of *Aspergillus flavus* is seen as being public health important because they are believed to produce aflatoxins which are among the most dangerous carcinogens to human (19). The moulds with the highest frequency of occurrence were *Aspergillus flavus, Aspergillus tamarii, Aspergillus niger, Aspergillus brunneoviolaceus*, and *Penicillium chrysogenum.* This was also reported by (20) and (21). It is likely that post-harvest infections and the storage structures greatly influence the mycoflora in storage (22). The two genera *Aspergillus* and *Penicillium* encountered are storage fungi while *Fusarium* is a field fungus (23:24).

The variations in the frequency and percentage occurrence in the stored products of grains can be as a result of the high moisture contents as shown in Table 3 (groundnuts (39%), maize (35%), rice (14%) and wheat (12%)) (25).

Table 5. Concentrations of Mycotoxins In Rice (Mg/Kg).

Mycotoxins	Concentration (µg/kg). Mean/standard deviation
Aflatoxin b_1	97.4 ± 2.500
Aflatoxin b_2	96.3 ± 0.100
Aflatoxing$_1$	97.5 ± 1.500
Aflatoxin g_2	94.2 ± 1.627
Ochratoxin a	100.5 ± 3.500
Citrinin	80 ± 1.700
Dihydrocitrinone	100.1 ± 2.510
Fumonisin b_1	85 ± 5.507
Fumonisin b_2	85 ± 1.1554
Fumonisin b_3	85 ± 2.081
Fumonisin b_4	85 ± 3.214
Zearalenone	99.2 ± 2.900
Deoxynivalenol	80 ± 1.154
Nivalenol	101.1 ± 2.066

Table 6. Concentrations of Mycotoxins In Wheat (Mg/Kg)

Mycotoxins	Concentration (µg/kg) . Mean/standard deviation
Aflatoxin b_1	84.3 ± 2.100
Aflatoxin b_2	86.1 ± 1.473
Aflatoxing$_1$	79.5 ± 2.500
Aflatoxin g_2	80.5 ± 0.763
Ochratoxin a	96.9 ± 1.300
Citrinin	43.9 ± 0.550
Dihydrocitrinone	115.5 ± 0.435
Fumonisin b_1	85 ± 5.507
Fumonisin b_2	85 ± 2.309
Fumonisin b_3	85 ± 3.214
Fumonisin b_4	85 ± 1.154
Zearalenone	100.1 ± 0.950
Deoxynivalenol	80 ± 0.577
Nivalenol	60 ± 2.00

Table 7. Concentrations of Mycotoxins In Groundnuts (µg/kg).

Mycotoxins	Concentration (µg/kg). Mean/standard deviation
Aflatoxin b_1	81.5 ± 0.763
Aflatoxin b_2	69 ± 2.000
Aflatoxing$_1$	65.3 ± 2.04
Aflatoxin g_2	62.2 ± 1.101
Ochratoxin a	86.6 ± 1.026
Citrinin	98.8 ± 3.002
Dihydrocitrinone	97.1 ± 2.510
Fumonisin b_1	85 ± 3.214
Fumonisin b_2	85 ± 1.154
Fumonisin b_3	85 ± 2.516
Fumonisin b_4	85 ± 1.527
Zearalenone	74.2 ± 0.642
Deoxynivalenol	80 ± 0.577
Nivalenol	88.2 ± 1.509

Table 8. Concentrations of mycotoxins in maize (µg/kg).

Mycotoxins	Concentration (µg/kg). Mean/standard deviation
AFLATOXIN B_1	58.2 ± 1.509
AFLATOXIN B_2	60.8 ± 1.750
AFLATOXING$_1$	84.7 ± 1.750
AFLATOXIN G_2	67.8 ± 1.400
OCHRATOXIN A	87.8 ± 2.052
CITRININ	27.1 ± 2.050
DIHYDROCITRINONE	147.3 ± 4.000
FUMONISIN B_1	85 ± 2.081
FUMONISIN B_2	85 ± 1.527
FUMONISIN B_3	85 ± 2.081
FUMONISIN B_4	85 ± 1.527
ZEARALENONE	85 ± 2.081
DEOXYNIVALENOL	80 ± 2.309
NIVALENOL	72.9 ± 2.066

Table 9. Concentrations Of Mycotoxins From Stored Products Of Grains (Mg /Kg)

GRAINS	CONCENTRATION OF MYCOTOXINS (µg/kg). MEAN/STANDARD DEVIATION
RICE	1286.3 ±29.689
WHEAT	1166.8 ± 0.901
GROUNDNUTS	1142.9 ± 10.488
MAIZE	1111.6 ± 9.810

Table 10. Quantifications of mycotoxins in µg/kg .

Mycotoxins	CONCENTRATION (µg/kg). MEAN/STANDARD DEVIATION
Fumonisins	1350 ± 10.000
Aflatoxins	1265.3 ± 1.327
Citrinin (dihydrocitrinone)	709.8 ± 1.039
Nivalenol (deoxynivalenol)	642.2 ± 1.900
Ochratoxin a	371.8 ± 1.616
Zearalenone	358.5 ± 2.500

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