Int. j. adv. multidisc. res. stud. 2023; 3(4):648-652

Received: 11-06-2023
Accepted: 21-07-2023

International Journal of Advanced Multidisciplinary Research and Studies

ISSN: 2583-049X

Molecular Characterization of Mycotoxin Producing Moulds and Mycotoxins Determination from Stored Grains and Legumes

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Abstract
Cereals and legume are among the staple food sources for people living in developing countries. 160 samples of the stored rice, maize, wheat and groundnuts were randomly collected from 3 different markets. They were stored for a period of 4 months in different packaging materials and analysed for the presence of mycotoxigenic moulds and mycotoxins production. 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 as well as 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 grains and legumes 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 grains and legumes were mainly contaminated with *Aspergillus* species and contained different mycotoxins of public health importance. The need for proper harvest and storage of grains and legumes cannot be overemphasized.

Keywords: *Aspergillus* Spp., Liquid Chromatograph Tandem Mass Spectrometry, Mycotoxins, Stored Grains and Legumes

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]. 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. 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 [4]. 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 [4]. Drying quickly and evenly remains the best way of preventing mycotoxigenic moulds that produce mycotoxins [5].

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 3 markets were stored in four different storage materials (sack, polyethene, plastic containers and metal containers) for four months at ambient temperature in a dry environment. Thirty grams (30 g) each of the stored samples were labeled and transported immediately to laboratory and kept in cool place prior to mycological analysis [6].

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 [7]. 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 [8].

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 [9].

Molecular Characterization of Moulds

The DNA of mould isolates were extracted using deoxyribonucleic acid extraction kit protocol. The extracted DNA was amplified using polymerase chain reaction (PCR) amplification protocol described by [10].

Sequencing Protocol

PCR products were cleaned using Exosap Protocol, sequenced using the Nimagen Brilliant dye Terminator cycle sequencing kit [10]. 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 [11].

Phylogenetic Tree Analysis

The obtained nucleotide sequence was analyzed using software, the geneious software version 4.0 [12].

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 [13].

Results and Discussions

The various moulds that were isolated from the stored grains and legumes 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 species and Penicillium spp. isolated from the stored grains and legumes.

Table 1: Cultural and microscopic characteristic of identified isolates

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

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.

Table 2: Sequence identity of various moulds

S. No	Sequence ID	Percentage (%)	NCBI Match	Isolates
1	NR111041.1	99	*Aspergillus flavus* NR135325	*Aspergillus flavus*
2	NR138279.1	97	*Aspergillus brunneoviolaceus* NR138279	*Aspergillus brunneoviolaceus*
3	AY373852.1	91	*Aspergillus niger* AY373852	*Aspergillus niger*
4	NR138306.1	99	*Penicillium chrysogenum* MH793845	*Penicillium chrysogenum*
5	AF004929.1	100	*Aspergillus tamari* MN339986	*Aspergillus tamarii*

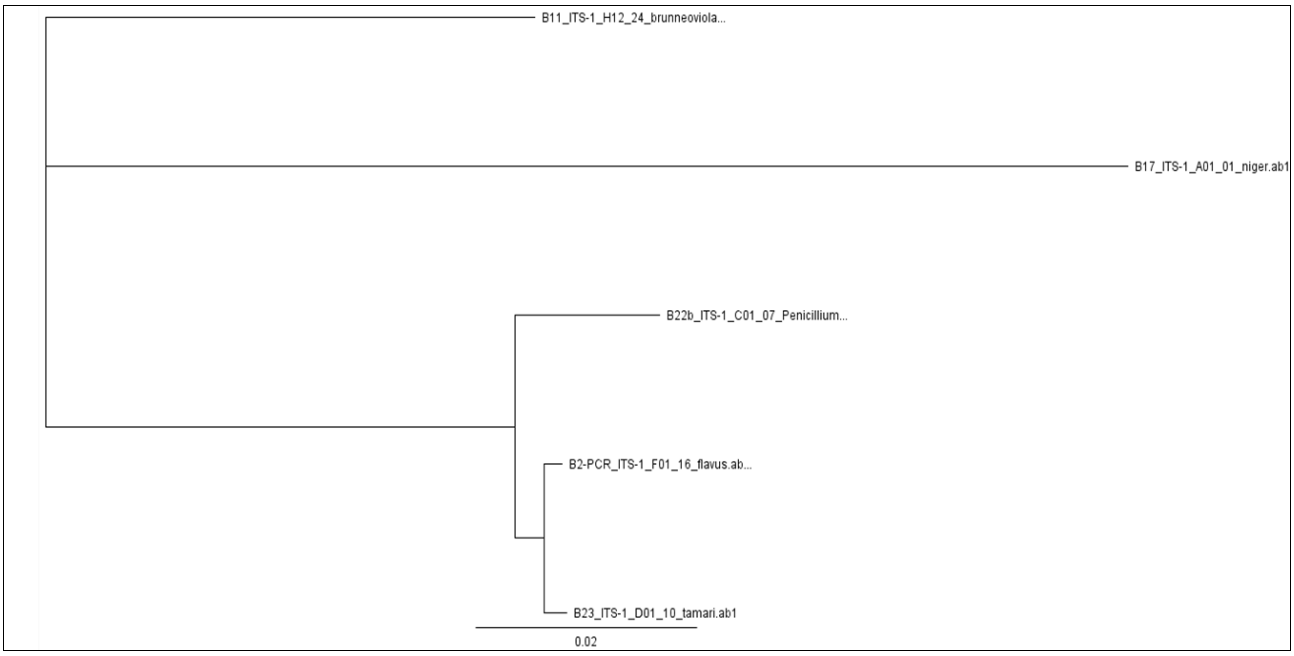

Fig 1: The Phylogenic tree constructed using the geneious software version 4.0 [12]

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%			

The grains and legumes (rice, maize, wheat and Groundnut) analyzed had different types of moulds as shown in (Table 2). The identified moulds were Aspergillus flavus, Aspergillus tamarii, Aspergillus niger, Aspergillus brunneoviolaceus, and Penicillium chrysogenum. These results were similar to the work of [14]. The most frequent genus isolated was Aspergillus with four different species namely Aspergillus flavus, Aspergillus tamarii, Aspergillus niger, Aspergillus brunneoviolaceus. Result in Table 3 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 their presence in stored grains and legumes can be detrimental to human health [15].

Table 3 result also showed that the frequency and percentage occurrence of moulds from different grains and legumes, with A. flavus (60%) from maize and A. niger from wheat (60%) predominating. This finding was similar to those reported by [16]. 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 [17]. The moulds with the highest frequency of occurrence were Aspergillus flavus, Aspergillus tamarii, Aspergillus niger, Aspergillus brunneoviolaceus, and Penicillium chrysogenum. Aspergillus species have great affinity for cereals were also reported by [18]. It is likely that post-harvest infections and the storage structures greatly influence the mycoflora in storage [19]. The two genera Aspergillus and Penicillium encountered are storage fungi while Fusarium is a field fungus [20]. The variations in the frequency and percentage occurrence in the stored grains and legumes can be as a result of their high moisture contents.

Fig 1 shows the phylogenic tree, showing relationship among various species of moulds isolated.

Table 4: Concentrations of mycotoxins in rice (µg/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 5: Concentrations of mycotoxins in wheat (µg/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 6: 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 7: 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 8: Concentrations of mycotoxins from stored grains and legumes (µg /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 9: 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

This study showed that Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2, Ochratoxin A, Citrinin, Dihydrocitrinone, Fumonisin B1, Fumonisin B2, Fumonisin B3, Fumonisin B4, Zearalenone, Deoxynivalenol and Nivalenol were detected from the stored grains and legumes. It was observed that Aflatoxin was the most frequent occurring mycotoxins detected from 160 samples analysed. Rice (1286.3 ± 29.689 µg/kg) contained the largest amount of the mycotoxins, followed by wheat (1166.8 ± 0.901 µg/kg), then groundnuts (1142.9 ± 10.488 µg/kg) while maize (1111.6 ± 9.810 µg/kg) had the least concentration of mycotoxins.

The four stored grains and legumes exceeded the maximum permissible limit for Aflatoxin B1, Aflatoxin B2, Aflatoxin G1, Aflatoxin G2 (0-40 µg/kg in food), Ochratoxin A (0-50 µg/kg in food), Citrinin and Dihydrocitrinone (0-100 µg/kg in foods), but did not exceed the limits for Fumonisin B1, Fumonisin B2, Fumonisin B3, Fumonisin B4 (0-1000 µg/kg in food), Zearalenone (0-1000 µg/kg in food) and Deoxynivalenol and Nivalenol (500-2000 µg/kg in food) [21].

The results showed that the predominant mycotoxin was Aflatoxin, which was seen relatively on all grain samples of rice, maize, wheat and groundnut. This agreed with the Research done over the years by [22]. Rice had the highest levels of aflatoxin, fumonisins and ochratoxin A [23].

Aflatoxin contamination is mainly reported in maize, peanuts and their products; fumonisin contamination in maize and maize products [24]. From the work of [3], showed that aflatoxins in groundnuts and groundnut-based products exceeded the United States of America and Nigeria Minimum permissible limit. According to [25] various mycotoxins like aflatoxins (AFs), fumonisins (FBs), ochratoxin A (OTA), citrinin (CIT), trichothecenes (deoxynivalenol (DON) and nivalenol (NIV)) and zearalenone (ZEN) were found in cereal, legume and their products in Nigeria. They used LC-MS/MS Liquid chromatography–tandem mass spectrometry method to analyze for the presence of mycotoxins. This agreed with this research work that cereals contain the above mentioned

mycotoxins when analyzed with the LC-MS/MS Liquid chromatography–tandem mass spectrometry method [13].

Acknowledgement
I wish to acknowledge the sponsorship of Tertiary Education Fund (TETFUND) Imo State University, Owerri for the scholarship given to me to carry out this research work.

Conclusion
The stored grains and legumes were mainly contaminated with Aspergillus species. Mycotoxins of public health importance were detected and some were above the permissible acceptable limit and has directly been linked to carcinogenic and neurological effects to human health. Therefore, the need for proper harvest and storage of grains and legumes cannot be overemphasized.

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