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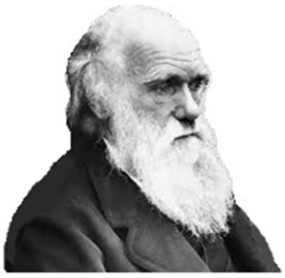

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Determination of mycotoxin producing moulds using culture and non-culture characterization and proximate composition of stored grains and legumes in Imo State, Nigeria

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ABSTRACT

Cereals and legumes are the major food sources for people in a developing country. Four grains and legumes (rice, maize wheat and groundnut) stored for 2 - 4 months in different packaging materials. These samples were randomly selected from three different markets. They were assessed for the presence of mycotoxin producing moulds and for the 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 used to analyze both the qualitative and quantitative occurrence of mycotoxin fungal metabolites. Proximate composition was determined using the method of Association of official Analytical chemist. The moulds isolated and identified culturally were *Aspergillus niger, Aspergillus flavus, Aspergillus* spp., *Aspergillus tamarii Penicillium chrysogenum, Fusarium* spp., *Rhizopus stolonifer, Rhizopus nigricans* and *Mucor* spp. The percentage occurrence of non-culturally 18S rRNA gene sequence dominant mould species identified were *Aspergillus flavus* (46%) followed by *Aspergillus tamarii* (23%), *Aspergillus niger* (18%), and *Penicillium chrysogenum* (9%) while the least was (4%) *Aspergillus brunneoviolaceus*. The Phylogenic tree was constructed by using the geneious software version 4.0. Aflatoxin, ochratoxins, fumonisin, deoxynevalenol and zearalenone were the different mycotoxins detected in stored grains and legumes. Ochratoxin A had the highest concentration of 371.8 ± 7.878 while Deoxynevalenol had the least concentration of 320 ± 4.617. Different values for Moisture Content, Crude Protein, Crude Fibre, Ash, Carbohydrate and Energy Value were determined. Groundnut 558.74 ± 279.37 had the highest energy value while Wheat 315.08 ± 157.54 had the least energy value. Grains and Legumes are essential for good health. There is a strong need to devise good storage condition for stored grains and legumes to avoid mycotoxigenic moulds contamination.

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***Keywords*:** *Aspergillus* spp., Mycotoxins, Phylogenetic tree, Proximate composition, Stored grains and legumes

1. INTRODUCTION

In Africa, Rice (*Oryza sativa*) Maize (*Zea mays*) Wheat (*Triticum aestivum*) and Groundnut (*Arachis hypogaea*) are essential food crops. They provide needed nourishment to the body. [1]. Moulds seen in stored grains and legumes can be classified as "field fungi" and the "storage fungi". Store fungi include all species of *Aspergillus, Penicillium* and *Fusarium*. Common genera of moulds include: *Fusarium, Mucor, Penicillium, Aspergillus, Cladosporium, Rhizopus, Trichoderma, Trichophyton and Stachybotrys* [2]. Moulds are dangerous to human health because they produce poisonous mycotoxins, which contaminate grains and legumes. The presence of mycotoxins in grains and legumes reduce their market value. Some factors that encourage fungal growth and proliferation include: Moisture content, Hydrogen ion concentration, temperature, presence of oxygen and carbondioxide, availability of nutrients, presence of preservatives and solute effects [3]. The detrimental effect caused by stored fungi include spoilage of grains, change in colour of grains, nutrient deficiency, difficulty in germination, high temperature, change in taste and aroma of grains, development of mycotoxins. The presence of mycotoxins causes reduction in quantity and quality of grains and legumes during storage. This lowers their market value. Mycotoxins are carcinogenic and mutagenic as well as detrimental to health [4]. Drying remains the best way of preventing storage fungi. Drying should be done quickly and evenly immediately after harvest to the critical moisture level of 0.7 [5]. Proximate composition determines the Moisture Content, Crude Protein, Crude Fibre, Ash, Carbohydrate and Energy value of stored grains and legumes.

The Proximate composition will help to determine the nutritional value of stored grains and legumes [6]. This study reports on the cultural characterization, molecular characterization of moulds associated with stored grains and legumes as well as the frequency of occurrence of moulds isolates in different zones of Imo State Nigeria as well as the mycotoxin determination, both qualitative and quantitative and proximate composition of stored grains and legumes.

2. MATERIALS AND METHODS

Sample collection and Preparation

Whole grains/fine powder of rice, maize, wheat and groundnut obtained from the three 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 (30g) of stored grains and legumes were labelled and transported immediately to laboratory and kept in cool place prior to mycological analysis. A total of two hundred and ten (210) samples were randomly collected following the method of [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 0.1 ml of lactic acid 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 (0.1 ml) of suspension was inoculated onto the freshly prepared surface dried media and incubated at 25 ± 2 °C a period of 7 days for mould growth. Moulds grown on media were subculture on various media [9] for further characterization and identification.

Enumeration, Morphological and Microscopic identification

A total cell count of each isolate was done on a counter using this formula [10]. The isolated moulds were identified based on colonial morphology and microscopic examination. The moulds were mounted on a clean grease slide, flooded with lacto phenol-cotton blue stain to determine mould structures. Microscopically, moulds were identified on the basis of spore characteristics, pigmentation and septation [11].

Molecular identification of bacterial isolates

Mould isolates were subjected to Deoxyribonucleic acid (DNA) extraction using Zymo research fungi DNA mini prep extraction kit. The qualitative estimation of genomic DNA was done using agarose gel electrophoresis. The Agarose gel electrophoresis determines the qualitative estimation of genomic DNA on a 0.8% -1% agarose gel, Mix 3 µl genomic DNA with 2 µl loading dye. Run for about one hour at 100v and view gel image using gel documentation system or transilluminator. The DNA obtained was amplified using polymerase chain reaction analysis. 1µl each of forward and reverse primers, internal transcribe space 1 and 4 (ITS 1 AND ITS 4), ITS 1 FORWARD PRIMER 5' –TCC GTA GGT GAA CCT GCG G-3' and the ITS 4 REVERSE PRIMER 5' –TCC TCC GCT TAT TGA TAT GC 3' The optical concentration of primers used was between 0.1 and 0.6 µM. This will ensure adequate yields of DNA amplicons and 18S rRNA sequencing method was done to characterize fungal isolates. Fragments were sequenced using the Nimagen Brilliant Dye terminator cycle sequencing kit and prepared according to manufacturer's instruction [12].

Basic Local Alignment Search Tool Nucleotide (BLASTn)

The sequenced data obtained were uploaded to the database of National center for biotechnology information (NCBI) to identify corresponding organisms [13].

Phylogenetic analysis

The obtained nucleotide sequence was analysed using software, the geneious software version 4.0 [14].

3. ANALYSIS OF MYCOTOXINS USING LIQUID CHROMATOGRAPH /TANDEN MASS SPECTROMETRY

A multimycotoxin method based on LC-MS/MS was applied to investigate the occurrence of mycotoxin fungal metabolites. Samples of Rice, maize, wheat and groundnut from the 3 geo political zones in Owerri were analyzed. Samples were homogenized and carefully kept in slant glass bottle and stored at 2-8 °C until further analysis, the qualitative and

quantitative analysis of mycotoxins (Aflatoxin, Ochratoxin, Fuminisin, Deoxynevalenol and Zearalenone) [15].

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) was added. Extraction, dilution, and analysis were performed as described by [14]. Detection and quantification were performed with a QTrap 4000 LC-MS/MS System (Applied Biosystems, Foster City, CA) equipped with a Turbo Ion Spray electrospray.

Ionization (ESI) source and an 1100 Series HPLC System from Agilent, Waldbronn, Germany. Chromatographic separation were performed at 25 °C on a Gemini C18 column, 150 × 4.6-mmi.d., 5-µm particle size, equipped with a C18 4 × 3-mm-i.d. security guard cartridge (all from Phenomenex, Torrance, CA, US). Both eluents contain 5 mM ammonium acetate and composed of methanol/water/acetic acid 10: 89 : 1 (v/v/v; eluent A) or 97 : 2 : 1 (eluent B), respectively. After an initial time of 2 min at 100% A, the proportion of B was increased linearly to 100% within 12 min, followed by a hold-time of 3 min at 100% B and 4-min column reequilibration at 100% A. The flow rate of 1 mL/min ESI-MS/MS was performed in the multiple reaction monitoring (MRM) modes both in positive and negative polarities in two separate chromatographic runs per sample by scanning two fragmentation reactions per analyte [15].

4. DETERMINATION OF PROXIMATE COMPOSITION

Proximate composition of the stored grains and legumes were determined in triplicates except for carbohydrate contents which were determined by difference.

The method of Association of official Analytical chemist was used to determine the moisture contents, Crude Protein, Crude Fibre, Carbohydrate and Energy Value [6].

Determination of moisture content

Procedure: The oven method was used. 3gms of each sample was weighed into a dried crucible. The samples were dried in a moisture extraction oven at 105 °C for 3 hours. The dried samples were cooled in desiccators and reweighed. This process was repeated until a constant weight is obtained. The difference in weight before and after drying was recorded as moisture content [6].

Determination of Ash content

Procedure: Two grams of each sample was weighed into a crucible and heated in a moisture extraction oven for three hours at 100 °C before being transferred into a muffle furnace at 550 °C until it turned white and free of carbon. The sample was removed from the furnace, cooled in desiccators and reweighed. The weight of the residual was calculated as ash content expressed in percentage [6].

Determination of crude protein

Procedures: The Protein content of the sample was determined by Kjeldahl method. The total nitrogen content was determined and multiplied with 6.25 to obtain the protein content.

One and half gram ($1^1/_2$) of each sample was mixed with 10mls of concentrated sulphuric acid. AR grade (Analytical Reagent grade) in Kjedadl digestion flask. A table of selenium catalyst was added to it and the mixture digested (heated) under a fume cupboard (digestion) until a clear line seen. The acid and other reagent was also digested but without sample forming in the blank control. All the digest was carefully transferred 100 ml volumetric flask using distilled water that was made up to mark in the flask. A 100 ml protection of each digestion mixed with equal volume/amount of 45% NaOH solution in Kjeldahl distilling unit/apparatus. The mixture was distilled and the distilled was collected into 10 ml of 4% boric acid solution containing three (3 drops) of mixed indicator. These was added |(bromocresil green and methyl red). A total of 100 ml distillate was collected and titrated against 0.02H (H_2S0_4) solution [6].

Determination of fat content

Procedure: The fat content of the samples was determined by the continous solvent of the extraction method using a soxhlet apparatus. Two grams of the samples was loosely wrapped with a filter paper and put into the thimble, fitted to a flask which has been cleaned dried and weighed. The flask contains 120 ml of petroleum spirit. The round bottom flask in soxhlet extraction unit was slowly heated for 5 hours. The heating was stopped and the thimbles with the spent samples will be kept and weighed later. The difference in weight was received as a mass of fat and was expressed as percentage of the sample [6].

Determination of crude fiber.

This was determined by A.O.A.C method (2016). Five grams (5.0g) of each sample was defatted (during fat analysis). The defatted sample was washed with several portions of hot boiling water using a two-field muslin cloth to trap the particle. The washed sample was carefully transferred quantitatively back to the flask and 20 ml of 1.25% NA0H solution was added to it again. The samples was boiled for 30 minutes and washed as before with hot water. Then they were carefully transferred to a weighted porcelain crucible and dried in the oven at 105 °C for 3 hours. After cooling in desiccators, they were reweighed (w_2) and then put in a muffle furnace and burn at 55 °C for 2 hours until they become ash again, they were cooked in a desiccators and reweighed [6].

Determination of carbohydrate

Procedure: The carbohydrate content was calculated by difference as the nitrogen free extractive (NFE), a method determined by A.O.A.C (2006). The NFE, were represented with carbohydrates = 100% - (a+b+c+d+e).

Where

A = protein

B = fat

C = fiber

D = ash

E = moisture [6].

5. STATISTICAL ANALYSIS

Statistical analyses were carried out using statistically available software (SPSS). The one way analysis of variance (ANOVA) was used to compare the mean occurrence of various organisms in the sample at 95% level of confidence.

6. RESULTS AND DISCUSSION

Moulds isolated from the stored grains and legume were characterized morphologically and microscopically. They were identified further by sequencing of 18S rRNA gene using ITS1 and ITS4 primers. Moulds were isolated from all the samples. Table 1 shows the mould species, *Aspergillus flavus*, *Aspergillus tamarii*, *Aspergillus niger*, *Aspergillus brunneoviolaceus*, and *Penicillium chrysogenum* isolated from the stored grains and legume.

Table 1. Cultural and microscopic characteristics of cultured based identified isolates.

Cultural	Microscopic	Isolates
Colonies are usually greenish or yellow brown in colour	Hyphae are septate	*Aspergillus* spp.
Colonies are green in colour	Hyphae are septate	*Aspergillus flavus* (Green *Aspergillus*, S Morphotype)
Black colonies are usually seen.	Black spores , septate hyphae	*Aspergillus niger* (Black *Aspergillus)*
Rusty brown or dark brown	Conidia head with long chain of conida,	*Aspergillus tamarii*
Blue green or shades of green colonies	Branched septate hyphae	*Penicillium chrysogenum* (Green *Penicillium)*
Pale or bright colour conidia	Septate hyphae	*Fusarium* spp.
White colonies becoming gray-brown	Rhizoids and stolons present	*Rhizopus stolonifer*
Colonies are black	Hyphae present are stolons, rhizoids and branching sporangiophores	*Rhizopus nigricans*
White, gray to black colonies in old culture	Hyphae branched	*Mucor* spp.

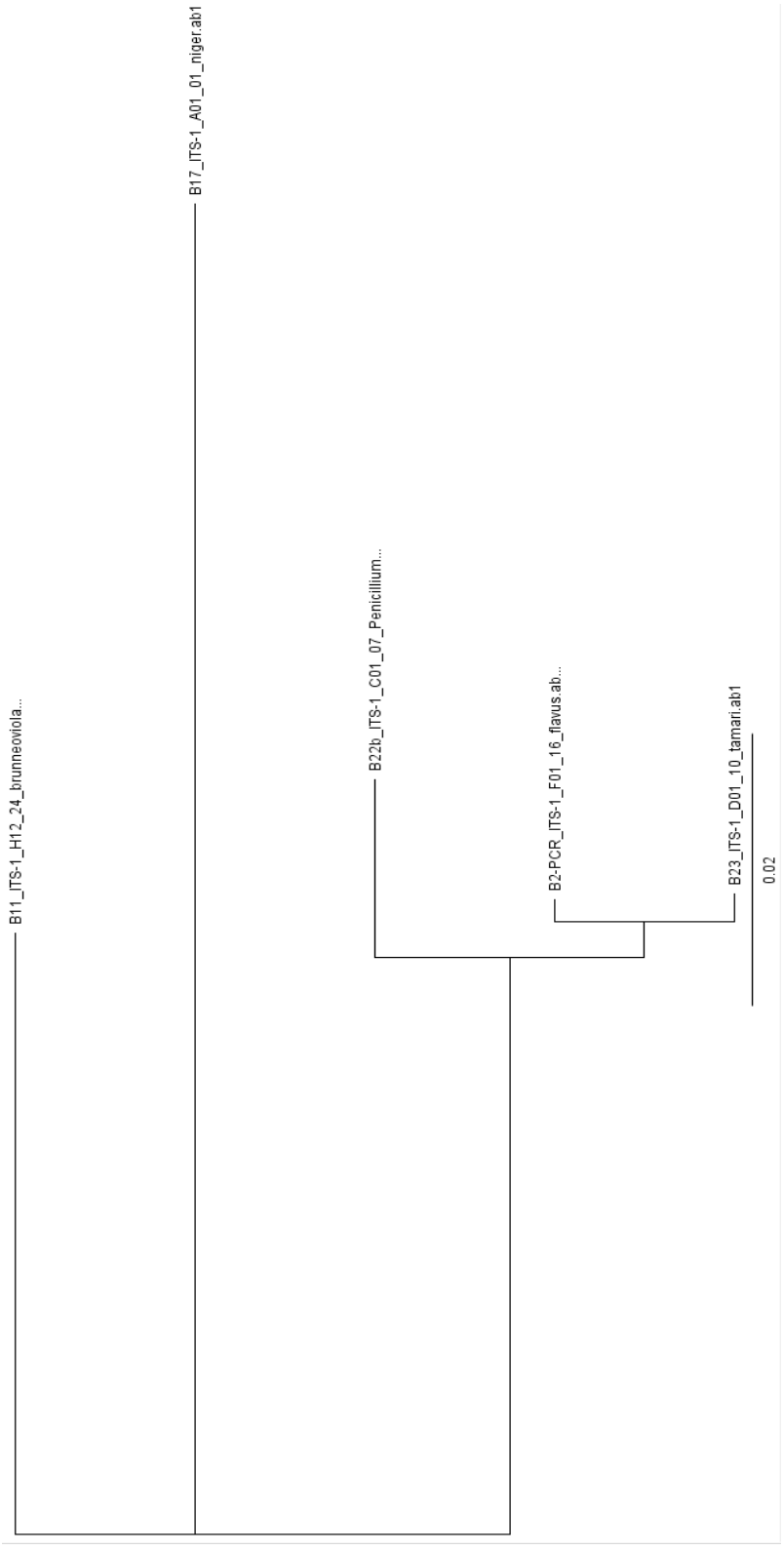

Figure 1. The Phylogenic tree was constructed by using the geneious software version 4.0.

Table 2. Non cultured based (18S rRNA gene sequence) identity of various moulds.

S/N	SEQQUENCE ID	PERCENTAGE (%)	NCBI MATCH	ISOLATE
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 tamarii* MN339986	*Aspergillus tamarii*

Table 3. Isolates from different zones in Imo State.

Isolates	Wheat			Maize			Rice			Groundnut			Total
	Owerri	Orlu	Okigwe	Owerri	Orlu	Okigwe	Owerri	Orlu	Okigwe	Owerri	Orlu	Okigwe	
S. morphotype	0	0	0	1	0	0	0	0	0	9	17	0	27
Black *Aspergillus*	0	0	2	4	0	2	2	0	4	7	37	0	58
Fusarium	1	6	-	19	23	44	-	19	4	9	-	5	130
Green *Aspergillus*	3	1	-	39	3	36	6	37	3	87	11	94	320
Aspergillus Tamarii	-	-	-	-	-	-	-	-	-	-	1	-	1
Green Penicillium	-	-	-	11	-	1	4	-	-	1	-	-	17
Other Genera	-	7	-	-	-	-	3	-	1	-	-	-	11
Total	4	14	2	74	26	83	15	56	12	113	66	99	564
	20			183			83			278			

Table 4. Frequency of occurrence of isolates from stored grains and legumes among the three different geo-political zones in Imo State.

Samples	No. of Isolates in Owerri	Percentage Occurrence (%)	No. of Isolates in Orlu	Percentage Occurrence (%)	No. of Isolates in Okigwe	Percentage Occurrence (%)	Total	Percentage Occurrence (%)
Wheat	4	2	14	9	2	1	20	4
Maize	74	36	26	16	83	42	183	32
Rice	15	7	56	34	12	6	83	15
Groundnut	113	55	66	41	99	51	278	49
Total	206	37%	162	29%	196	34%	564	100%

Table 5. Qualitative analysis of mycotoxins in stored grains and legumes.

MYCOTOXINS	Aflatoxin B_1	Ochratoxin A	Fumonisin B_1	Deoxynevalenol	Zearalenone
Rice	+	+	+	+	+
Maize	+	+	+	+	+
Wheat	+	+	+	+	+
Groundnut	+	+	+	+	+

Table 6. Quantitative analysis of mycotoxins in stored grains and legumes.

MYCOTOXINS	Aflatoxin B_1 (ppb)	Ochratoxin A (ppb)	Fumonisin B_1 (ppb)	Deoxynevalenol (ppb)	Zearalenone (ppb)
Rice	97.4 ±2.500	100.5 ± 3.500	85 ±5.507	80±1.154	99.2 ±2.900
Maize	58.2 ± 1.509	87.8 ± 2.052	85 ±2.081	80 ±2.309	85 ± 2.081
Wheat	84.3 ± 2.100	96.9 ± 1.300	85 ±5.507	80 ±0.577	100.1 ± 0.950
Groundnut	81.5 ±0.763	86.6 ± 1.026	85 ± 3.214	80 ± 0.577	74.2 ± 0.642

Table 7. Proximate composition of stored grains and legumes.

SAMPLE	% MC	% CP	% FAT	% CF	% ASH	% CHO	(KCAL) EV
RICE	6.88 ± 3.32 6.85 ± 3.31	7.69 ± 3.73 7.71 ± 3.77	0.74 ± 0.02 0.71 ± 0.01	2.33 ± 1.15 2.37 ± 1.18	0.79 ± 0.01 0.76 ± 0.01	81.57 ± 29.71 81.60 ± 29.80	363.70 ± 181.76 363.63 ± 181.71
MAIZE	8.49 ± 4.20 8.45 ± 4.15	12.22 ± 6.08 12.24 ± 6.14	9.85 ± 0.04 9.82 ± 0.01	3.10 ± 1.53 3.13 ± 1.56	2.57 ± 0.01 2.53 ± 0.00	63.77 ±28.48 63.83 ±28.53	392.61 ± 196.30 392.66 ± 196.33
WHEAT	7.65 ± 3.82 7.68 ± 3.86	12.44 ±6.21 12.41 ±6.20	1.56 ± 0.01 1.58 ± 0.02	7.69 ± 3.84 7.64 ± 3.82	7.84 ± 0.00 7.88 ± 0.01	62.82 ± 30.06 62.80 ± 28.9	315.08 ± 157.54 315.06 ± 157.52
GROUND NUT	7.26 ± 3.65 7.23 ± 3.60	25.92 ±12.90 25.94 ±12.94	49.14 ±0.02 49.11 ±0.01	7.83 ± 3.91 7.87 ± 3.93	6.65 ± 0.01 6.61 ± 0.00	3.20 ± 1.61 3.24 ± 1.62	558.74 ± 279.37 558.71 ± 279.36

Key: MC - Moisture Content, CP - Crude Protein, CF - Crude Fibre, CHO - Carbohydrate, (KCAL) EV – Energy Value.

Among the various stored grains and legume namely: rice, maize, wheat and groundnuts. Groundnuts (49%) had the highest Percentage occurrence (number of isolates), followed by maize (32%), rice (15%) while the least was wheat (4%). There was a significant difference ($P < 0.05$) among the stored grains and legume in Imo State. The high nutritional (energy value) of groundnuts may account for the highest Percentage occurrence of isolates on groundnuts. Owerri had the highest number of isolates (37%), followed by Okigwe (34%) and Orlu (29%), but there was no significant difference ($P < 0.05$) among the isolates on the different geo-political zones in Imo state.

Aspergillus flavus was the highest isolate observed from the stored grains and legume *Aspergillus*, *Fusarium* and *Penicillium* were the genus observed. The research done by [16] observed that both the maize and groundnut samples were contaminates with at least one types of mycotoxigenic moulds like *Aspergillus*, *Penicillium* and *Fusarium*. That research also suggested that the highest occurring mould genera were *Aspergillus*, *Penicillium* and *Fusarum* in that order. *Aspergillus flavus* was the highest contaminating mould. These findings were similar to this research work. [17] observed that moulds contamination requires high moisture content.

According to [18] discovered that *Aspergillus* species have great affinity for cereals. Seven *Aspergillus* species namely *Aspergillus flavus, Aspergillus parasiticus*, *Aspergillus ochraceus, Aspergillus niger, Aspergillus fumigatus, Aspergillus wentii* and *Aspergillus ruber*. *Aspergillus* genera are known to produce aflatoxins, ochratoxins, fumonisins, gliotoxin, aspertoxins as well as other harmful mycotoxins was observed by [19]. Aflatoxin was detected in maize and groundnuts from the study of [20]. This agreed with this research having aflatoxin as the major toxin produced from grain and legume samples of rice, maize, wheat and groundnut. The Research work also done over the years by Nigerian Stored Products Research Institute (NSPRI) has revealed the presence of aflatoxin in Nigerian products like groundnut and livestock feed, maize, millet and sorghum [21]. (Oyeniran, 1978; Opadokun and Ikeorah 1979). [22] Suggested that traditional drying of cereals on bare floor may account for its high fungal contamination and also observed that Poor storage practices may lead to the production

of mycotoxin. Proximate composition of stored grains and legumes showed that Groundnut 558.74 ± 279.37 had the highest energy value while Wheat 315.08 ± 157.54 had the least energy value. Moisture content was highest in maize and least in rice. Crude protein was highest in groundnut and least in rice. Fat was highest in groundnut and least in rice. Crude fibre was highest in groundnut and least in rice. Ash was highest in wheat and least in rice. Carbohydrate was highest in rice and least in groundnut. The consumption of legumes reduces the risks of developing cardiovascular diseases like cancers. Legumes also help maintain a good body weight [23]. According to [24] wheat has been reported to be low in essential nutrient such as lysine and has also been implicated for the incidence of celiac disease: a systemic immune-mediated disorder caused by the ingestion of gluten-containing grains. There is need to blend wheat with groundnuts to improve its nutritional value. This agreed with this study since groundnuts had the highest energy value while wheat had the least energy value. Groundnuts are used to fortify wheats and other grains to increase their functional and nutritional values, inorder to improve health [25].

7. CONCLUSION

The cultured and non-cultured based method of isolation yielded *Aspergillus* species as the predominant mould from stored grains and legumes. While the frequently occurring mycotoxin was aflatoxin. Proximate composition had groundnut with the highest energy value and wheat had the least energy value. This necessitates that proper care should be taken while storing grains and legumes because high prevalence of mycotoxigenic moulds remains a threat to healthy life. Proper harvest and storage of grains will improve and maintain a healthy nation.

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