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Identities and Prevalence of *Aspergillus* Species on *Phaseolus vulgaris* (Bean) Seeds Sold in Ihiala, Anambra State, Nigeria

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ABSTRACT

Ascertaining the real characteristics, identity and prevalence of microorganism is the major step in controlling foodborne pathogens. This study was carried out to determine the identities and prevalence of *Aspergillus* species associated with bean seed samples sold in Ihiala, Anambra State. A total of 90 representative bean seed samples of different varieties (*mesugar, Oloka, honey, potasco white, potasco brown, Sokoto* bean, *Iron* white, *Iron brown* and *soya* bean) were randomly collected from different shops and open markets in Ihiala Local Government Area, Anambra State, and screened for the presence of Aspergillus species using spread plate technique and surface plating of 25 seeds per Petri dish on sterile poured plates. The isolates obtained were characterized and identified using macroscopic, microscopic and molecular characteristics. It was observed that 32 (35.56 %) and 14 (14.44 %) of the studied bean seed samples recorded *Aspergillus* species for surface and internal contaminants. The surface of *Oloka* bean seeds (70 %/30 %) and inner part of Sokoto bean seeds (50%/50%) were most contaminated. *Aspergillus flavus* strain HUS6 (AFHUS6), *Aspergillus niger* strain HG48 (ANHG48), *Aspergillus niger* strain HUS1 (ANHUS1), *Aspergillus tubingiensis* strains EM-CN1 (ATEM-CN1), *Aspergillus aculeatus* strain AN5 (AAAN5) and *Aspergillus awamori* strain DN-SN2 (AW DN-SN2) were isolated from the bean seed samples. AN HUS1 was mostly significant (P<0.05) in both surface and internal contamination of the studies bean seed sample. Thus, this study has shown that AFHUS6, ANHG48, ANHUS1, ATEM-CN1, AAAN5and AW DN-SN2 were isolated from the studied bean seed samples of which ANHUS1 was most prevalence mostly on the surface of Oloka bean and internally in Sokoto bean.

INTRODUCTION

Beans are the most important grain legumes for human consumption in the world. Beans represents one of the total world production of pulse (19.3 Mt/ year; Norena-Ramirez *et al.,* 2014).) and total production exceeds 23 million metric tonnes (MT) of which 7 million MT are produced in Latin America and central Africa where it is the staple food for many people due to its energy, protein, dietary fiber and minerals content (Norena-Ramirez *et al.,* 2014). Beans come in many varieties of shapes, sizes and colours, from pinto to pink, black and white, interesting enough, despite this diversity in colour and size, the wild and domestic beans belong to the same species, as do all of the colourful varieties of beans, which are believed to be the result of a mixture of population bottlenecks and purposeful selection (Lerner, 2009).

Fungi are the most frequent pathogenic agent and represent the major threat to this crop since they attack the root parts and destroy the proper functioning of the plant in taking up water and other nutrients (Nana *et al*., 2015). The diseases can be caused by a single soil – borne pathogens, resulting in disease complexes (Nana *et al.,* 2015). *Rhizoctonia solani, Fusarium oxysporum, F.* spp. *Phaseoli, F. solani F.* spp. *Phaseoli, Macrophomina phaseoli, Sclerotium rolfsii, Meloidogyne* spp., *Aspergillus* spp. (Domijan *et al*., 2015;Nana *et al.,* 2015) are among the major pathogens known to impact bean production in many countries in Latin America and Africa. Seeds are the vehicles of transmission of several fungi and frequently introduce new pathogen in exempt areas, so that the integration between seed health and germination tests is recommended to control seed transmitted diseases (Francisco and Usberti, 2008).

Fungi are known to produce one or more toxic secondary metabolites in bean seeds. The presence of these toxins in foods and food products is a serious health hazard to consumers (Betina, 2012). Seed borne fungi pathogens are the principal producers of mycotoxins associated with fungal growth on crops in the field and in storage (Betina, 2012). It is widely acknowledged that *Aspergillus* and *Penicillium* species are the most important mycotoxin-producing fungi in tropical countries, seen mostly among adults in rural populations with a poor level of nutrition for whom common beans is the staple food. (Tulpule and Bhat, 2012).

Although there have been a number of investigations related to the bean, little is known about the identities and prevalence of *Aspergillus* spp. on bean seeds, therefore this study sought to evaluate the identities and prevalence of *Aspergillus* spp. on bean seeds.

MATERIALS AND METHODS

Study Area: These study was done in Ihiala Local Government Area (L.G.A.), Anambra State, located at latitudes 5.85°N and longitudes 6.85°E on the Southeast part of Nigeria. Ihiala is predominantly a low lying region on the elevational plain of Manu river with all parts at 146 meters above sea level Ihiala has rainforest vegetation with two seasonal climatic conditions. There are rainy season and dry season which is characterized by harmattan between December and February. Ihiala is characterized by the annual double maxima rainfall with a slight drop in August break. The annual total rainfall is about 1600 mm with relative humidity of 80% at dawn. Ihiala has minimum daily temperature of 18°C, annual minimum and maximum temperature ranges are about 22°C and 34°C respectively (Jim *et al*., 2009).

Sample Collection: A total of ninety samples of bean seeds were collected randomly, from different shops and open markets in Ihiala Local Government Area (L.G.A.), Anambra State. Sampling was performed manually from different bags and basins, such that the bean seeds were collected from different parts of the bags and basins. The samples were aseptically pooled and mixed properly and formed one cup of the bean seeds in sterile nylon bag, then the bean seeds were taken for analysis. The samples were carefully labeled and then kept in a disinfected cooler, to maintain its temperature and stability of the number of the isolates. The samples were transported to the laboratory for analysis.

Isolation of the Fungi Isolates: This was done using the method of Suleiman and Omafe (2013). Each sample was shared into two groups. First group was aseptically soaked into distilled water for 30 minutes, and the second group was disinfected by soaking for 1 minute in 1% Sodium hypochloride and washed three times with distilled water, and then soaked in the distilled water for 30 minutes. A 0.1 ml aliquot from the first group was plated on Sabouraud Dextrose Agar (SDA) containing chloramphenicol antibiotics (0.05%). Seeds from the second group was placed at the rate of 25 seeds Per Petri dish containing 20 ml of SDA supplemented with chloramphenicol antibiotics (0.05%).These were incubated at room temperature (30±2ºC) for 5 days. The fungi obtained were aseptically sub cultured on SDA containing chloramphenicol antibiotics (0.05%) and incubated at room temperature (30±2°C) for 5 days.

Identification of Fungal Isolates: The fungal isolates were identified to the genus/species level based on macroscopic, microscopic and molecular characteristics of the isolates obtained from pure cultures (Watanabe, 2002).

Prevalence of the Isolates: The occurrences of each identified isolate were carried by determining the number of times the isolate occurred in the samples, and also estimated the percentage of the occurrence using the formula below:

$$\text{Percentage Occurrence} = \frac{\text{Total No of Each Isolate} \times 100}{\text{Total No of Isolates}}$$

Statistical Analysis: The occurrences of the isolates were presented in percentages, and pairwise comparison of occurrence each isolate was carried out using student “T” test.

RESULTS

A total ninety (90) samples of bean seeds were screened for the presence of *Aspergillus* species, out of these, 32(35.56%) were positive to *Aspergillus* species surface contamination whereas 13 (14.44%) were positive to *Aspergillus* species for internal contamination as show in Table 1. The occurrence of *Aspergillus* species was seen most on *Oloka* bean (70%) seed samples for surface contamination and in Sokoto bean (50%) seed samples for internal contamination. Iron white bean (10%) seed samples showed least occurrence of *Aspergillus* species for surface contaminant. Honey bean (0.00%), Iron white (0.00%) bean and Soya bean (0.00%) samples did not record any occurrence of *Aspergillus* species for internal contaminants.

The isolates were characterized macroscopically using their initial and their final appearances on Sabouraud Dextrose Agar (SDA), colour on the reverse side of the plate and colouring of the mycelium as shown in Table 2. Isolates Y1, Y2, M, Q and R have almost similar morphology, dark brown to black mycelia and belong to the group of *Aspergillus* known as Negri section. Isolates Y1 and Y2 were almost identical except the slight different in their mycelia appearances. Isolate R was also similar to isolates Y1 and Y2 but differ slightly in its initial appearances on SDA, colour on the reverse side of the plate and colour of the mycelium. Similarly, isolates Q and M showed slight variations in their initial appearances and colours of the reverse side of the plates, Isolate X showed clear variation from isolates Y1, Y2, M, Q and R: and possessed distinct features of the group of *Aspergillus* known as flavi section. The characteristics features of the hyphae, conidia, conidiophores, vesicle, sterigmata and metulae covering were basically used for the microscopic features of the fungal isolate as shown in tables isolates Y1, Y2, M, Q and R had almost similar microscopic features of Negri section group of *Aspergillus*. The five isolates differ in the diameter of their vesicles: with slight variation in the colour of their conidia. Isolate Q showed clear distinction of being uniseriate whereas other isolates were biseriate. Isolates Y1 and Y2 have similar features but differ from isolates M, Q and R by having brownish condidiophore. Isolate X was clearly different from isolates Y1, Y2, M, Q and R. Isolate X showed short conidiophores, yellowish green conidia, three- quarter metulae covering and columnaric conidia head, which are features of flavi section of *Aspergillus*.

The qualities of nucleic acids (DNA) extracted from isolates x, Y1, Y2, M, Q and R were within the stipulated range (1.80-1.90). Purity of nucleic acids (DNA) was determined by calculating the ratio of the absorbance A_{260}/A_{280} as shown in Table 4. The gel characteristics of polymerase chain reaction (PCR) of the nucleic acids (DNA) extracted from isolated X, Y1, Y2, M, Q and R is shown in figure 1. The regions coding internal transcribed spacer rDNA (ITS1-1.58 ITS2), B-tubulin (Ben A) and Calmodulin (Cam) were amplified and electrophoresed using 1.5% agarose. The photograph of the gel reviewed clear amplification of the selected regions for sequencing of the isolates. The amplicons were cleaned (figure 2) and then sequenced. The amplicons generated from nucleic acids (DNA) extracted from the isolates were cleaned and re-electrophoresed as shown in figure 2. The sequencing of the amplified regions of isolates X, Y1, Y2, M, Q and R showed 100% identities of each of the isolates (Table 5).

The study revealed the presence of *Aspergillus flavus* strain Hus6 (isolates x), *Aspergillus niger* strain HG 48 (isolate Y1), *Aspergillus niger* strain HUS 1 (Isolate Y2), *Aspergillus tubingiensis* strain Em-CN1 (Isolate M), *Aspergillus aculeatus* strains AN5 (Isolate Q) and *Aspergillus awamori* strain DN-SN2 (Isolate R). *Aspergillus niger* strain Hus 1 was mostly significant (P<0.05) in both surface and internal contamination of the studies bean seed sample whereas *Aspergillus tubingiensis* strain Em-CN1 and *Aspergillus aculeatus* strain AN5 were not detected for internal contaminant. For surface contaminants, *Aspergillus tubingiensis* strain EM-CN1 (ATEM-CN1) *A. aculeatus* strain AN5 (AA AN5) and *A. Awamori* strains DA-SN2 (AWDA-SN2) were not seen in Mesugar bean samples *A. flavus* strain HUS 6 (AF HUS 6), ATEM-CN1 and AA AAN5 were not seen in Honey bean samples, AF HUS6 and ATEM-CN1 were not seen in *Potasco* white bean sample, AA AN5 was not seen in *Potasco* brown bean samples, *Aspergillus niger* strain HG48 (ANHG48), AFHUS 6 and ATEM-CN1 were not seen in Sokoto bean sample, ATEM-CN1 and AA An5 were not seen in Soya bean samples, and *Aspergillus niger* strain HUS 1 (AN HUS1) was only seen in Iron white bean samples. For internal contaminants, AN HUS1 was only seen in *Mesugar, Potasco* white and *Potasco* brown bean samples, ATEM-CN1 and AA AN5 were not seen in *Oloka* and Sokoto bean samples, ANHUS1 and ANHG48 were only seen in Iron brown bean sample, and no organism was seen in Honey, Iron white and Soya samples.

Table 1: Bean seed samples that were positive to *Aspergillus* species

N = 10				
Samples	Surface contaminant		Internal contaminant	
	Positive (%)	Negative (%)	Positive (%)	Negative (%)
Mesugar	4 (40.00)	6 (60.00)	1 (10.00)	9 (90.00)
Honey Bean	3 (30.00)	7 (70.00)	0 (0.00)	10 (100.00)
Oloka Bean	7 (70.00)	3 (30.00)	3 (30.00)	7 (70.00)
Potasco white	4 (40.00)	6 (60.00)	1 (10.00)	9 (90.00)
Potasco Brown	3 (30.00)	7 (70.00)	1 (10.00)	9 (90.00)
Sokoto Bean	2 (20.00)	8 (80.00)	5 (50.00)	5 (50.00)
Iron White	1 (10.00)	9 (90.00)	0 (0.00)	10 (100.00)
Iron Brown	6 (60.00)	4 (40.00)	2 (20.00)	8 (80.00)
Soya Bean	2 (20.00)	8 (80.00)	0 (0.00)	10 (100.00)
Total	32 (35.56)	58 (64.44)	13 (14.47)	77 (85.56)

Table 2: Macroscopic characteristics of the fungal isolates

Parameter	Isolates x	Isolates Y1	Isolates Y2	Isolate M	Isolate Q	Isolate R
Initial Appearance on SDA (2-3 days)	Yellow	White	White to yellow	White pitchy	Colourless	Colourless to creamy
Final Appearance on SDA (5 days and above)	Yellow to dark green	Park brown	Carbon black	Black with white edges	Dark brown	Chocolate black
Reverse colour colony growth	Pale yellow	Pale yellow	Pale yellow	Pale	Yellow	dull yellow to reddish brown
Rate	Moderate to rapid	Rapid	Rapid	Rapid	Moderate to rapid	Moderate to rapid
Texture of the colony	Velvety	Powdery/ Velvety	Velvety /powdery	Rough	Rough	Tough/Velvety
Colour Mycelium	Yellowish green	Park brown	Black	Black with white edges	Dark brown	Chocolate

Table 3: Microscopic characteristics of the fungal isolates

Parameter	Isolates x	Isolates Y1	Isolates Y2	Isolate M	Isolate Q	Isolate R
Nature of hyphae	Septate	Septate	Septate	Septate	Septate	Septate
Colour of conidiophores	Hyaline	Hyaline (Brownish)	Hyaline (Brownish)	Hyaline (Colourless)	Hyaline (Colourless)	Hyaline (Colourless)
Texture of conidiophores	Rough	Smooth	Smooth	Smooth	Smooth	Smooth
Length of conidiophores	Short	Long	Long	Long	Long	Long
Seriation (sterigmata)	Biseriate	Biseriate	Biseriate	Biseriate	Uniseriate	Biseriate
Diameter of vesicle (mm)	24	38	45	42	64	30
Shape of Vesicle	Globose	Globose	Globose/Subglobose	Globose	Globose	Globose
Shape of conidia	Ellipsoidal	Globular/Ellipsoidal	Ellipsoidal	Globular/Ellipsoidal	Ellipsoidal	Ellipsoidal
Colour of Conidia	Yellowish green	Dark brown	Black	Black	Dark brown	Chocolate
Conidia head	Columnar	Radiate	Radiate	Radiate	Radiate	
Texture of conidia	Spiny	Finely winkled	Finely wrinkled	Finely winkled	Spiny to wrinkled	Finely wrinkled
Metula covering	Nearly entire vesicle	Entire	Entire	Entire	Entire	Entire

Table 4: Quality of nucleic acid (DNA) used for study

Sample	Concentration of Nucleic acid (mg/ml)	A_{280}	A_{260}	260/280
X	72.10	0.44 03	0.7970	1.81
Y1	88.20	0.5366	0.9970	1.86
Y2	88.60	0.5312	0.9986	1.88
M	79.80	0.5170	0.9410	1.82
Q	66.70	0.4356	0.7840	1.80
R	84.60	0.5286	0.9780	1.85

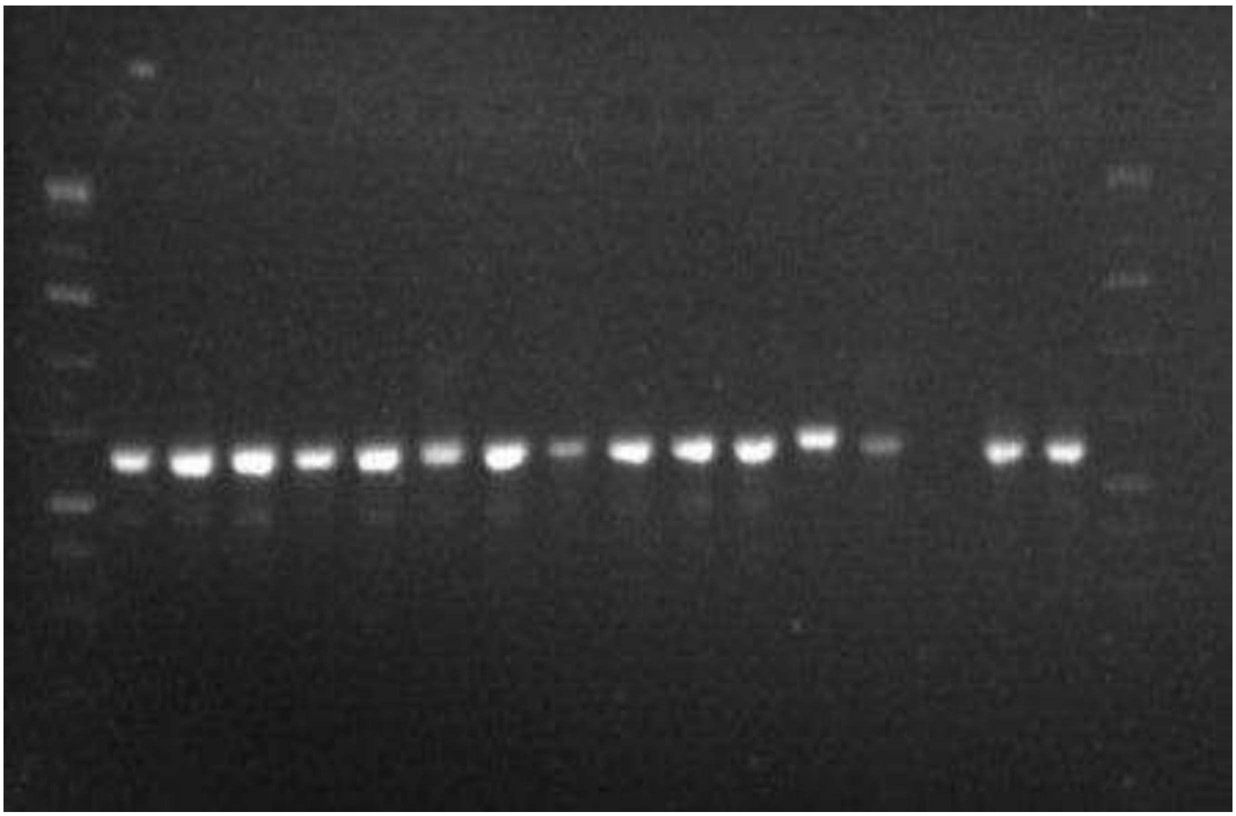
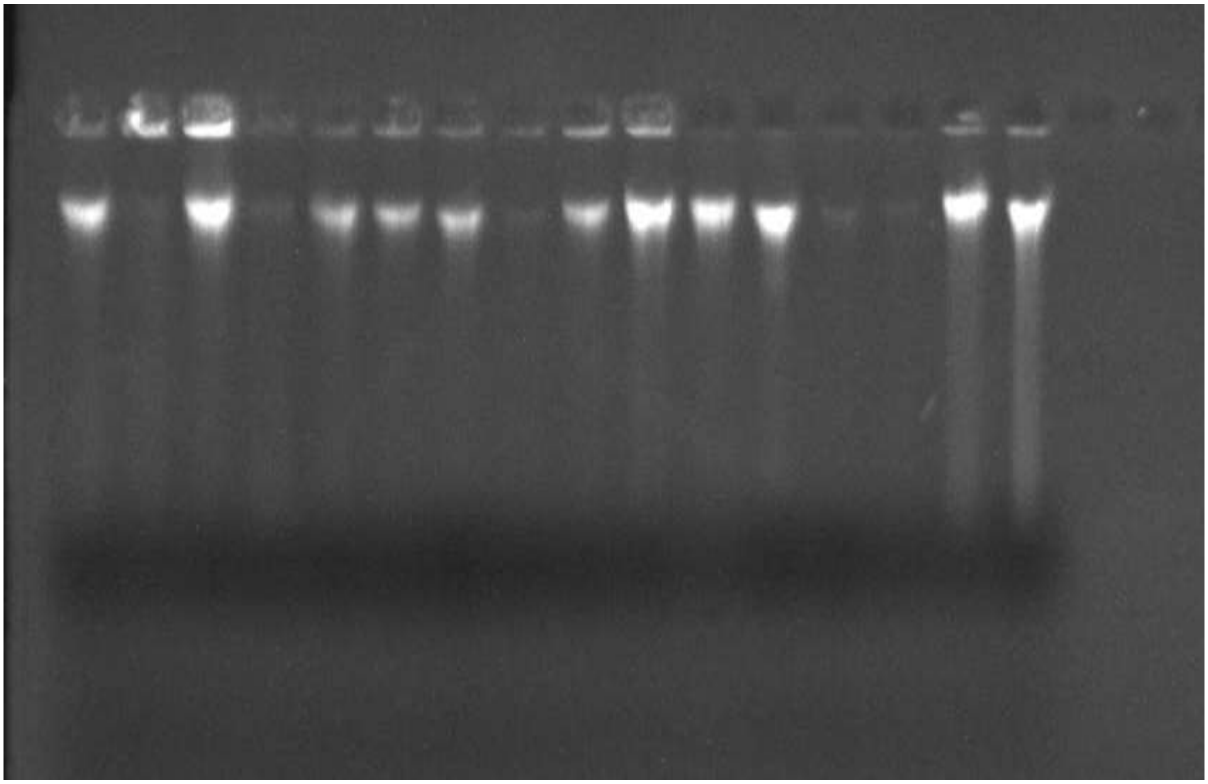


Figure 1: Gel electrophoresis showing PCR product for the 6 samples on 1.5 % Agarose gel where T = Marker



Y1 Y2 Q Q X X R R M R M

Figure 2: Gel characteristics of the amplicons for sequencing

Table 5: Molecular identities of the isolates

Isolate	Max score	Total score	Query cover	GCP (E-value)	Identity	Accession Number	Description
X	719	719	100%	0%	100%	MF 163443.1	*Aspergillus flavus* strain HUS 6
Y1	701	701	100%	0%	100%	KX 099668.1	*Aspergillus niger* strain HG48
Y2	832	832	100%	0%	100%	MF 163441.1	*Aspergillus niger* strain HUS 1
M	832	832	100%	0%	100%	KY 509548.1	*Aspergillus tubingiensis* strain EM-CN1
Q	793	793	100%	0%	100%	KU 527791.2	*Aspergillus aculeatus* strain AN 5
R	785	785	100%	0%	100%	KY 509551	*Aspergillus awamori* strain DA-SN2

Table 6: Prevalence of *Aspergillus* species in the studied bean seed samples

Sample	Surface Contaminants						Internal contaminants					
	AF HUS 6 (%)	AN HG48 (%)	AN HUS 1 (%)	AT EM-CN 1 (%)	AA AN 5 (%)	AW DA-SN2 (%)	AF HUS 6 (%)	AN HG48 (%)	AN HUS 1 (%)	AT EM-CN 1 (%)	AA AN 5 (%)	AW DA-SN2 (%)
Mesugar	1 (1.49)	2. (2.99)	2. (2.99)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (0.00)	0 (5.88)	0 (0.00)	0 (0.00)
Honey bean	0 (0.00)	1 (2.99)	3 (4.48)	0 (0.00)	0 (0.00)	1 (1.49)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Oloka Bean	2 (2.99)	0 (0.00)	5 (7.46)	1 (1.49)	2 (2.99)	3 (4.48)	1 (5.88)	1 (5.88)	2 (11.76)	0 (0.00)	0 (0.00)	1 (5.88)
Potasco White	0 (0.00)	3 (4.48)	1 (1.49)	0 (0.00)	3 (4.48)	1 (1.49)	0 (0.00)	0 (0.00)	1 (5.88)	0 (0.00)	0 (0.00)	0 (0.00)
Potasco Brown	1 (1.49)	1 (1.49)	3 (4.48)	1 (1.49)	0 (0.00)	2 (2.99)	0 (0.00)	0 (0.00)	1 (5.88)	0 (0.00)	0 (0.00)	0 (0.00)
Sokoto Bean	0 (0.00)	0 (0.00)	2 (2.99)	0 (0.00)	1 (1.49)	2 (2.99)	1 (5.88)	1 (5.88)	3 (17.65)	0 (0.00)	0 (0.00)	2 (11.76)
Iron White	0 (0.00)	0 (0.00)	1 (1.49)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Iron Brown	3 (4.48)	2 (2.99)	4 (5.97)	1 (1.49)	2 (2.99)	3 (4.48)	0 (0.00)	1 (5.88)	1 (5.88)	0 (0.00)	0 (0.00)	0 (0.00)
Soya Bean	1 (1.49)	1 (1.49)	2 (2.99)	0 (0.00)	0 (0.00)	1 (1.49)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)
Total	8 (11.94)	12 (17.91)	23 (34.33)	3 (4.48)	8 (11.94)	13 (19.40)	2 (11.76)	3 (17.65)	9 (52.94)	0 (0.00)	0 (0.00)	3 (17.65)

AF HUS 6 – *Aspergillus flavus* strain HUS 6; *Aspergillus niger* strain HG48; *Aspergillus niger* strain HUS 1; *Aspergillus tubingiensis* strain EM-CN1, *Aspergillus aculeatus* strain AN5; *Aspergillus awamori* DA-SN2

DISCUSSION

The presence of *Aspergillus* species in the studied been seed samples could be traced from poor handling, management practices, transportation of the bean seed samples and sanitary conditions distributed to the bean seed samples. Similar findings were reported by many researchers (Immersed *et al.,* 2002; Maciorowski *et al.,* 2007; Iheukwumere *et al.,* 2017). Many studies have shown that bean seeds contaminated by pathogenic *Aspergillus* species could contribute to human food-borne illness through the food-human chain. This shows that preparation of bean seeds for human consumption requires microbiological safety regulations to escape contamination by pathogenic microorganisms. Similar deduction was drawn by different researchers (Davies and Wales, 2010; Chowdhuri *et al.,* 2011; Fredrick and Huda, 2011).

The variation in the occurrence of *Aspergillus* species from different types of been seeds observed in this study could be attribute to the nature, texture, water activity and proportion of nutrients in the bean seeds. Similar deductions were drawn by many researchers (Maciorowski *et al.,* 2007; Iheukwumere *et al.,* 2017).

The presence of *Aspergillus flavus* strain HUS6, *Aspergillus niger* strain HG48, *Aspergillus niger* strain HUS1 *Aspergillus tubingiensis* strain EM-CN1, *Aspergillus aculeatus* strain AN5 and *Aspergillus awamori* strain DA-SN in the studied bean seed samples supported the occurrence of *Aspergillus* species in the bean seeds, and this corroborates with the report of many researchers (Barakat, 2004; Damijan *et al;* 2014). Traditionally, the laboratory defection of *Aspergillus* species has relied on non-selective and selective enrichment media and subsequent subculture on selective media. The introduction of molecular techniques provides more sensitive and rapid technique for detecting these fungi. The genes that code translation elongation factor-1; calmudulin, β-tubulin and internal transcribed spacer rDNA region (ITS1-1.585-ITS2) were amplified and used as barcode for the fungal identification. These genes were also reported by other researchers (Samson *et al.,* 2007; Varga *et al.,* 2007).

The highest counts of *Aspergillus niger* strain Hus 1 recorded in the studied bean seed samples could be attributed to human activities during processing, transportation and the storage of the bean seeds. Similar deduction was made by Banford and Adebanjo (2011).

CONCLUSION

From this study, it was observed that *Aspergillus flavus* strain HUS6, *Aspergillus niger* strain H948, *Aspergillus niger* strain HUS1, *Aspergillus tubingiensis* strain EM-CNI, *Aspergillus aculeatus* strain AN5 and *Aspergillus awamori* strain DA-SN2 were isolated from the studied bean samples, of which *Aspergillus niger* strain HUSI was the most predominant. This study suggest proper handling and good hygienic measures must go in parallel with good management practices to minimize the occurrence of the fungi in the studied samples.

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