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ORIGINAL RESEARCH

The distribution and mycotoxigenicity of fungal isolates of stored maize grains from five agro-ecological zones of Nigeria

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ABSTRACT

Seventy composite samples of stored maize grains collected from farmers' storage structures in five agro-ecological zones of Nigeria (AEZs): Sudan Savanna (SS), Northern Guinea Savanna (NGS), Southern Guinea Savanna (SGS), Derived Savanna (DS) and Humid Forest (HF) were examined for fungal contamination. The isolates were further assessed for their abilities to produce mycotoxins in culture medium. The *Aspergillus* isolates found in the grains were *A. niger*-clade, *A. fumigatus* and *A. tamari*. The *Aspergillus* population (63.7–76.8%) was significantly higher than the population of other fungal genera in the grains excluding grains from the HF zone where the *Penicillium* species (51.4%) occurred in higher abundance of 51.4% to 35.03% of *Aspergillus*. In addition the *Fusarium* isolates were more abundant (19.4%; $p<0.05$) in the NGS zone than the other zones. Of the five *Fusarium* species (*F. semitectum*, *F. verticillioides*, *F. graminearum*, *F. proliferatum* and *F. nygamai)* isolated in the grains, *F. verticillioides* recorded a high occurrence of 100% in both the NGS and SS zones respectively. The isolates were found to produce aflatoxins, fumonisins, zearalenone and ochratoxin A in culture medium and *A. flavus* was the only aflatoxin-producing species among the *Aspergilli*. Stored maize grains in Nigeria thus contain an array of mycotoxigenic moulds which may increase the risk of mycotoxin exposure since environmental and storage conditions in sub-Saharan Africa favour mycotoxin production.

Keywords: Aflatoxin, Fumonisin, Maize, Fungi, Ochratoxin A, Zearalenone**.**

1.0 Introduction

Fungal and mycotoxin contamination of foods is an increasing issue of concern in sub-Saharan Africa (SSA). Several studies have shown that *Aspergillus*, *Fusarium* and *Penicillium* species and their toxic secondary metabolites are the primary contaminants of stored foods in SSA (Bankole and Adebanjo, 2003; Chilaka *et al*., 2012; Atanda *et al*., 2013). These moulds invade crops on the fields, colonise them, and are often transmitted from field to store where they proliferate under favourable environmental and poor storage (post-harvest) conditions which are characteristic of SSA countries (Cotty and Jaime-Garcia, 2007). Mycotoxins may be produced by moulds on foods and when ingested by humans or animals, produce diverse health effects ranging from carcinogenicity to immune suppression and deaths (CAST, 2003). The presence of toxigenic moulds and their mycotoxins in foods usually lead to direct loss of agricultural products and/or reduced income for farmers due to rejection of such commodities in international markets even though the foods pass through sales points in the local markets where little or no regulation/monitoring are enforced.

Maize, a staple crop widely cultivated in Nigeria and other parts of the world, remains a major focal crop for mycotoxigenic fungal attack (Aja-Nwachukwu and Emejuaiwe, 1994; Kpodo *et al*., 2000; Bankole and Mabekoje 2003; Hell *et al*.,

2003; Ezekiel *et al*., 2008; Atehnkeng *et al*., 2008; Kankolongo *et al*., 2009; Egbuta, 2011; Chilaka *et al*., 2012; Atanda *et al*., 2013; Mohale *et al*., 2013). Despite this, literature is deplete of information on the distribution of these fungal isolates in farmers' storage structures across the agro-ecological zones (AEZs) of the country. Since storage and handling practices vary from one AEZ to another there is a probability that variation in the fungal population of the stored grains can occur within the AEZs. The present work therefore aims at investigating the variations in the distribution of isolates of stored maize grains sampled from five Agro ecological zones of Nigeria

2.0 Materials and methods

2.1 Survey sites

Surveys were conducted between August 2011 and February 2012 in five out of the seven AEZs of Nigeria where maize is predominantly produced (Adetunji *et al*., 2014a): SS: Kano and Sokoto states, NGS: Kaduna state, SGS: DS: Ondo, Ekiti, Osun, Oyo and Nasarawa states and HF: Lagos and Ogun states. The geographical location, temperature and rainfall patterns of the zones had been documented by previous workers (Udoh *et al*., 2000; Atehnkeng *et al*., 2008).

2.2. Sampling and sample preparation

Sampling was carried out as described by Adetunji *et al.* (2014a). Seventy composite samples (3 kg each) were collected across the five AEZs: HF (n = 4), SGS (n = 11), NGS (n = 11), SS (n = 11) and DS (n = 33). The samples were kept in well labelled sterile polyethylene bags and transported to the laboratory for analysis. Each sample was hand-mixed, coarse-ground and allowed to pass through a No.14-mesh screen. Sub-samples of 500 g were taken from each lot, ground with a milling machine (Greiffenberger Antriebstenchnic, Germany) and further sieved with a 1-mm mesh. Sub-samples of 50 g were taken from the lots and kept in zip lock envelopes for fungal isolation.

2.3 Mycological analysis of maize grains

2.3.1 Isolation of fungi

Fungi were isolated from the 50 g sub-samples by the dilution plating technique (Samson *et al*., 1995). Ten grams of each sub-sample was diluted in 90 ml of 0.1 % sterile peptone water and the mixture vortexed for 2 min. Aliquots (0.1 ml) were inoculated by surface plating on ½ strength Potato Dextrose Agar (PDA) plates supplemented with 0.01% chloramphenicol. Isolations were made twice from each sub-sample in triplicate PDA plates. The first set was incubated at 30 ^{o}C for 3 days for enumeration of *Aspergillus* species while the second set was incubated at 25 ^{o}C for 7 days for enumeration of *Fusarium* and *Penicillium* species. Yeasts were enumerated from PDA plates of both sets.

2.3.2 Identification of isolated fungi

Colonies of the isolates that bore resemblance to *Aspergillus*, *Fusarium* and *Penicillium* species were transferred to full strength PDA, peptone-pentachloronitrobenzene agar (PPA) and water agar (WA; 20 g agar/l of distilled water) respectively for further characterization. The cultures on PDA were incubated at 30 ^{o}C for 7 days while those on PPA and WA were incubated at 25 ^{o}C for 7 days. Cultures of *Fusarium* were incubated under fluorescent light on a 12 h day/night schedule to initiate conidia which were subsequently used for single sporing. Single spores of the isolates were manipulated and incubated overnight on a WA plate at 25 °C. Germinated spores were then maintained on a modified Czapek Dox complete medium (CM) and stored at 4^{o}C until identification.

All isolates were identified on the basis of morphological characteristics and comparison with appropriate keys in literature. Isolates belonging to the *Aspergillus* section *Flavi* group and other sections such as *Fumigati* were identified to species level according to Klich (2002), Ehrlich *et al*. (2007) and Pitt and Hocking (2009) while all black isolates of *Aspergillus* were regarded as belonging to the section *A. niger*-clade (Pitt and Hocking, 2009). For the *Aspergillus* section *Flavi* group, data

obtained from morphological character assessment (macro: colony colour, morphology and size; micro: conidia morphology and size) of each isolate was matched with the aflatoxin production profile of the isolates on yeast extract (2%) sucrose (20%) agar as illustrated below. All identified *Aspergillus* isolates were further maintained on PDA slants by the single colony transfer technique at 4 ^{o}C.

The *Fusarium* isolates stored on CM were inoculated on Carnation Leaf Agar (CLA) for 14 days after which they were identified based on morphological characteristics exhibited on CLA (sporodochia and uniform macro conidia) under the Olympus BX51 Digital Microscopy, Olympus Optical Co., LTD, Japan and for pigmentation and colony morphology on PDA. Species identification was according to the taxonomic criterion of Leslie and Summerell (2006). Macro- and micro- characters of *F. verticillioides* and *F. semitectum* isolates were compared to those of *F. verticillioides* ATCC MYA 836 reference strain (Afolabi *et al*., 2007) and *F. semitectum* BUFC 059 (Ezekiel *et al*., 2013). The identified *Fusarium* isolates were then cultured on CM slants in a 4 ml vial, and stored at 4^{o}C. All *Penicillium* isolates were identified to the genus level (Samson *et al*., 2010) while the yeast isolates were stained with lactophenol cotton blue and observed under Olympus BX51 Digital Microscope (Olympus Optical Co., LTD, Japan) for cell shape, sporulation and characteristic vegetative reproduction.

2.3.3 Assessment of mycotoxigenic potential of fungal isolates

All isolates of *Aspergillus*, *Fusarium* and *Penicillium* were assessed for their ability to produce aflatoxins (AF), fumonisins (FB), zearalenone (ZEA) and ochratoxin A (OTA) in culture. Each isolate was sub-cultured on Yeast Extract Sucrose (YES) agar and incubated for 14 days at 28 ºC (Singh *et al*., 1991). At the end of the incubation period, 10 g of the culture medium was gently removed from the Petri dish and added to a vial containing 20 ml of dichloromethane for mycotoxin extraction. The mixture was homogenised, filtered through Whatman No. 1 filter paper and spotted on a two-dimensional Thin Layer Chromatographic (TLC) plate (Chilaka *et al*., 2012). A 20 µl aliquot of the filtrate from each sample was spotted alongside the mycotoxin standards: aflatoxin B_1 (AFB_1), AFB_2, fumonisin B_1 (FB_1), ZEA and OTA. After the double development of spotted plates, the plates were dried; plates for AF and OTA determination were viewed under UV light at wavelengths of 365 and 360 nm respectively. The fluorescing colour and retardation factor (Rf) value of the spots of the extracts were compared with those of the mycotoxin standards. In addition, the dried chromatographic plates for FB_1 and ZEA were sprayed with anisaldehyde reagent and diazotiseddianisidine respectively. Plates for FB_1 determination were further heated for 1 min at 120 ºC and then viewed under UV light at 313 nm while plates for ZEA were viewed at short wavelength of 260 nm. Plates that showed the desired colouration under UV light at the various wavelengths were qualitatively assessed and considered as positive (+) for the specific mycotoxin

2.4 **Statistical analysis**

All data were analyzed by SPSS® 16.0 (Windows version, SPSS, IL, USA). One way ANOVA was performed for the distribution of fungal species across the agro-ecological zones (AEZs). All means were tested for significance by the Duncan's Multiple Range Test at 95% confidence level.

3.0 Results

3.1 Fungal profile of maize grains

A total of 566 fungal isolates belonging to four genera: *Aspergillus* (*n* = 350), *Penicillium* (*n* = 151), *Fusarium* (*n* = 45) and *Saccharomyces* (*n* = 20) were isolated from the grains (Table 1). The population of *Aspergillus* (63.7–76.8%) was higher than the population of the other fungal genera of the grains in the AEZs except the HF zone where the *Penicillium* species (51.4%) were more abundant. Among the *Aspergillus* species isolated, *A. flavus* was the only aflatoxin-producing species and was the most abundant species in all the AEZs. The incidence

of *A. flavus* in the grains from the NGS (71.5%) and the SGS (75.4%) zones was significantly (p<0.05) higher than the grains from other zones. Following *A. flavus* in hierarchical succession was the *A. niger*-clade whose incidence in the DS, HF and SS zones was significantly (p<0.05) higher than the SGS and NGS zones while it was not isolated from the NGS zone. The incidences of *A. fumigatus*, *A. tamarii* and *S. cerevisae* in the grains were very low (≤5.0%) in the AEZs (Table 1).

The highest incidence (19.4%; p<0.05) *of Fusarium* isolates was found in maize grains from the NGS zone (Table 1) and the isolated *Fusarium* species were: *F. semitectum, F. verticillioides, F. graminearum, F. proliferatum* and *F. nygamai* (Table 2). *Fusarium verticillioides* was the most dominant *Fusarium* species in the AEZs and had an occurrence of 100% in the SS and NGS zones. In addition, *F. graminearum* was isolated only from maize grains from SGS zone (Table 2). Table 3 shows the diversity of mycotoxigenic moulds (*Aspergillus*, *Fusarium* and *Penicillium*) within the agro-ecological zones and the toxins (aflatoxins, fumonisins and zearalenone, ochratoxin A) produced by the isolates respectively. The aflatoxins were the most the most commonly contaminating mycotoxins in the AEZs

4.0 Discussion

Four fungal genera: *Aspergillus, Fusarium, Penicillium and Saccharomyces*; were identified in this study as contaminants of stored maize grains across the AEZs. These fungal genera with the exception of the yeast are known to widely contaminate stored maize (Aja-Nwachukwu and Emejuaiwe, 1994; Kpodo *et al*., 2000; Hell *et al.,* 2003; Kankolongo *et al.,* 2009; Chilaka *et al*., 2012; Mohale *et al*., 2013). The high incidence of *A. flavus* in the grains across the AEZs and its significantly higher occurrence in the grains than all other *Aspergillus* species including those belonging to the section *Flavi* agrees with previous reports from Nigeria (Bankole and Mabekoje 2003; Atehnkeng *et al*., 2008; Egbuta, 2011).

The common occurrence of *A. flavus* in Nigeria's AEZs is consistent with findings of Kankolongo *et al. (*2009) that *A.flavus* and *A.niger* clade were the most prevalent fungal isolates in Zambian maize. This suggests similarity of prevalent conditions such as the occurrence of *A. flavus* in the soil and plant debris (Horn and Dorner, 1999; Nesci and Etcheverry, 2002; Jaime-Garcia and Cotty, 2004). The plant debris usually acts as reservoirs of inoculums for infection of kernels in the field. This could be due to the occurrence of *A. flavus* in the soil and plant debris (Horn and Dorner, 1999; Nesci and Etcheverry, 2002; Jaime-Garcia and Cotty, 2004) which act as the reservoir of inoculums for infection of kernels in the field.

Table 1. Incidence of fungal species in stored maize grains from five agro-ecological zones of Nigeria.

	% Occurrence of fungal species in AEZ*				
Fungal species	NGS	SGS	DS	HF	SS
Aspergillus flavus	71.52^{a}	75.42^{a}	50.91^{b}	35.03^{c}	51.41^{b}
A. niger-clade	0.00^{b}	0.89^{b}	11.16^{a}	8.97^{a}	14.19^{a}
A. fumigatus	0.00^{a}	0.00^{a}	0.05^{a}	0.34^{a}	0.06^{a}
A. tamari	3.80^{a}	0.46^{b}	1.53^{a}	0.00^{b}	5.01^{a}
Fusarium spp.	19.41^{a}	6.82^{b}	9.32^{b}	4.23^{b}	0.56^{c}
Penicillium spp.	5.27^{c}	16.22^{b}	26.95^{b}	51.44^{a}	28.21^{b}
Saccharomyces cerevisae	0.00^{a}	0.18^{a}	0.08^{a}	0.00^{a}	0.56^{a}

*****AEZ**-** Agro-ecological zones: Northern Guinea Savanna (NGS), Southern Guinea Savanna (SGS), Derived Savanna (DS), Humid Forest (HF) and Sudan Savanna (SS).

Percentage occurrence values with different superscript alphabets in a row are significantly different (α = 0.05).

Table 2. Incidence of *Fusarium* species in stored maize grains from five agro- ecological zones of Nigeria

[a]AEZ	Incidence of *Fusarium spp.*	[b]*F. verticilliodes*	*F. semitectum*	*F. proliferatum*	*F. nygamai*	*F. graminearum*
SS	1/11	100[a]	0.00[c]	0.00[c]	0.00[b]	0.00[b]
NGS	9/11	100[a]	0.00[c]	0.00[c]	0.00[b]	0.00[b]
SGS	15/33	25[c]	25.00[a]	0.00[c]	0.00[b]	50.00[a]
DS	4/11	44.45[b]	11.11[b]	11.11[b]	33.33[a]	0.00[b]
HF	3/4	50[b]	0.00[c]	50.00[a]	0.00[b]	0.00[b]

[a]Agro-ecological zones: Sudan Savanna (SS), Northern Guinea Savanna (NGS), Derived Savanna (DS), Southern Guinea Savanna (SGS) and Humid Forest (HF).
[b]Percentage occurrence of *Fusarium spp*
Percentage occurrence values with different superscript alphabets in a columm are significantly different (α = 0.05).

The non-isolation of other aflatoxigenic members of *Aspergillus* section *Flavi* (unnamed taxon SBG, *A. parasiticus and A. parvisclerotigenus*) from the grains in this study contrasts previous reports by Atehnkeng *et al.* (2008) and Perrone *et al.* (2014) who found these three species in Nigerian maize in addition to the widely distributed *A. flavus* at very low frequencies.This may be attributed mainly to (1) the choice of isolation and characterization medium- PDA, used in our study instead of modified Dichloran Rose Bengal Agar (DRBA), a selective medium for isolation of *Aspergillus* and *Penicillium* species and 5/2 agar for differentiation of *Aspergillus* section *Flavi* species (Cotty, 1989; Diedhiou et al., 2011) and (2) the relatively scarce distribution of the species in maize.

The occurrence of *A. fumigatus* in the grains is in line with previous reports on maize in Nigeria (Makun *et al.*, 2010; Egbuta *et al.,* 2011). However, Makun *et al,*. (2010) found low occurrence of *A. fumigatus* (2.3%) in maize grain from markets and farmers' storage structures in Nigeria, Egbuta *et al.* (2011) and Chilaka *et al.* (2012) found high incidence of *A. fumigatus* (>40%) than *A. flavus* in commercial maize from South Africa and Nigeria. The high incidence of some fungal species across the AEZs and low incidence of others confirm that fungi that thrive or found to be scarce in a particular area are strongly determined by the prevailing climatic conditions (Wayne, 2007).

The incidence of *Penicillium* species was higher than that of *Fusarium* species across the AEZs except for the NGS zone. The high occurrence of *Penicillium* species in the AEZs contrasts the findings of Atehnkeng *et al*. (2008) and Chilaka *et al.,* (2012) who reported low incidences of *Penicillium* species in maize grains while it corroborated the reports of Egbuta (2011) who found more *Penicillium* species (57.9%) in stored maize grains than *Fusarium* species (47.4%). In spite of the relatively low occurrence of *Fusarium* species than *Penicillium* and *Aspergillus* species in the grains in this study, its wide distribution across the AEZs of Nigeria shows that *Fusarium* species is a regular contaminant of maize grains (Fandohan *et al*., 2005; Adejumo *et al*., 2007; Chilaka *et al*., 2012; Mohale *et* al., 2013). The occurrence of *F. verticillioides* as the most common *Fusarium* species isolated from the grains is in agreement with the work of Kankolongo *et al.,* 2009 and Chilaka *et al*., 2012. In addition, *F. nygamai* is reported for the first time in Nigerian maize grain as it was found in grains from the DS zone in contrast to previous studies by Adejumo *et al.*, (2007) and Ezekiel *et al.*, (2008) that reported only *F. graminearum*, *F. verticillioides*, *and F. proliferatum* in maize from South-Western Nigeria.

The significance of this study lies in the diversity of mycotoxigenic moulds found in Nigerian

maize grains. It is known that when moulds invade and colonise a suitable agricultural commodity such as maize, they utilize the available nutrients thus deteriorating the grains. The moulds may further liberate mycotoxins in the commodity depending on the mycotoxigenic potential of the moulds, condition and duration of storage of the commodity as well as the prevailing environmental factors. The incidence of mycotoxigenic *A*. *flavus* and *Fusarium* species in this study and the toxins (aflatoxins, fumonisins, zearalenone and ochratoxin A) produced by the isolates in the conditions of storage is a potential risk to the health of consumers of the grains. A major concern is the attendant health effects that could arise from consumption of these grains by Nigerians as mycotoxins are potent carcinogens, nephrotoxins and immune system toxicants (Bondy and Pestka, 2000; CAST, 2003). Besides, multi-mycotoxin exposure has recently been reported in Nigerian population (Ezekiel *et al.,* 2014 and Adetunji *et al.,* 2014b).

Stored maize grains in Nigeria are contaminated with propagules of mycotoxigenic moulds and possible additional risk may be cross-contamination of maize and groundnuts since both are mainly co-stored in rural areas of West Africa. Intervention strategies such as: (1) development of maize varieties that are resistant to a range of fungal infections and subsequent mycotoxin formation (2) reduction of wounds on the crop during cropping and harvesting or by insects (use of insecticides) and (3) harvesting of grains prior to onset of hot, dry climate conditions that may significantly increase infections by these fungi and (4) drying of maize cobs to a moisture content of about 13% after harvest prior to storage

Conclusion

The isolates were found to produce aflatoxins, fumonisins, zearalenone and ochratoxin A in culture medium and *A. flavus* was the only aflatoxin-producing species among the *Aspergilli*. Stored maize grains in Nigeria thus contain an array of mycotoxigenic moulds which may increase the risk of mycotoxin exposure since environmental and storage conditions in sub-Saharan Africa favour mycotoxin production.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Table 3: Mycotoxin Profile of Fungal Isolates from Five agro-ecological zones of Nigeria

AEZ	Toxin	Fungal Specie								
	Parameter	*A. flavus*	*A. niger*	*A. fumigatus*	*F. verticilloides*	*F. semitectum*	*F. proliferatum*	*F. graminearum*	*F. nygamai*	*Penicillium spp*
SS	Toxin tested	AFB_1,B_2,G_1,G_2	OTA	AFB_1,B_2,G_1,G_2	FB_1, ZEA	ND	ND	ND	ND	OTA
	Toxin produced	AFB_1,B_2,G_1,G_2	ND	ND	FB_1	ND	ND	ND	ND	ND
	Intensity	+, +, +, +	ND	ND	+	ND	ND	ND	ND	ND
NGS	Toxin tested	AFB_1,B_2,G_1,G_2	OTA	AFB_1,B_2,G_1,G_2,	FB_1, ZEA	ND	ND	ND	ND	OTA
	Toxin produced	AFB_1,B_2,G_1	ND	ND	FB_1, ZEA	ND	ND	ND	ND	ND
	Intensity	+, +, +	ND	ND	++, ±	ND	ND	ND	ND	ND
DS	Toxin tested	AFB_1,B_2,G_1,G_2	OTA	AFB_1,B_2,G_1,G_2,	FB1, ZEA	FB1, ZEA	FB1, ZEA	ND	FB1, ZEA	OTA
	Toxin produced	AFB_1,B_2,G_1,G_2	OTA	ND	FB1, ZEA	FB1, ZEA	FB1, ZEA	ND	FB1, ZEA	OTA
	Intensity	++, +, +, +	+	ND	+, ++	+, +	+, +	ND	+, +	++
SGS	Toxin tested	AFB_1,B_2,G_1,G_2	OTA	AFB_1,B_2,G_1,G_2,	FB1, ZEA	FB1, ZEA	ND	FB1, ZEA	ND	OTA
	Toxin produced	AFB_1,B_2,G_1,G_2	ND	ND	FB1, ZEA	ND	ND	ZEA	ND	OTA
	Intensity	++, +, +, +	ND	ND	+, ±	ND	ND	+	ND	+
HF	Toxin tested	AFB_1,B_2,G_1,G_2	OTA	AFB_1,B_2,G_1,G_2,	FB_1, ZEA	ND	FB_1, ZEA	ND	ND	OTA
	Toxin produced	AFB_1,B_2	ND	ND	FB_1	ND	FB_1	ND	ND	ND
	Intensity	+, +	ND	ND	++	ND	+	ND	ND	ND

++ Mycotoxigenic positive isolates with high fluorescence intensity under UV light

+ Mycotoxigenic positive isolates with moderate fluorescence intensity under UV light

± Mycotoxigenic positive isolates with weak fluorescence intensity under UV light

ND- Not Detected

HF- Humid Forest; DS- Derived Savanna; SS- Sudan Savanna SGS-Southern Guinea Savanna, NGS- Northern Guinea Savanna

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