Toxigenic *Aspergillus* section *Flavi* and aflatoxins in Dried Yam Chips in Oyo State, Nigeria

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Abstract: The distribution of aflatoxigenic *Aspergillus* and quantity of aflatoxins were determined in 40 dried yam chip samples collected from four markets in Ibadan, Nigeria. A control chip was prepared to assess the influence of storage duration on toxin accumulation. Two hundred and thirty-four isolates of the *Aspergillus* section *Flavi* group were recovered from 92.5% of the chips. *A. flavus* L-strain was the most occurring taxon (>60% incidence) while S_{BG} strain and *A. parasiticus* were the least predominant (<4.5% incidence). The relative proportion of toxigenic to atoxigenic strains varied across markets with significant (P<0.05) incidence of atoxigenic over toxigenic in 75% of the locations. The overall incidence for B- and G-aflatoxin contamination was 97.5% with aflatoxin B_1 having the highest concentration of 190µg/kg. About 67.5% of the chip samples violated the EC regulation on aflatoxin levels in food. Consequently, contaminating mould and toxin reduction strategies targeted towards the post-production storage methods should be considered since there was the highest positive correlation (r=0.99) for the influence of storage duration on aflatoxin concentration in the contaminated samples. This will ensure public health safety.
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1. Introduction

Yam (*Discorea* spp.) tubers are a very important food crop in West and Central Africa, especially in the areas from Nigeria to Cote d'Ivoire. This region alone produces more than 80% of world's production of yam with Nigeria being among the highest contributing states (FAO, 2005). It is consumed also in the South Americas, India and South-East Asia (Degras, 1986). The tuber which is rich in carbohydrate (Akissoe *et al*., 2001) is usually consumed in many different forms: as boiled tuber, fried tuber, pounded yam or boiled flour from dried chips. The dried chip and its flour (*elubo*) is one of the traditional transformation methods for processing and storage of yam since yam has been noted to be devastated by deteriogenic bacteria and fungi due to it high moisture content (Babajide *et al*., 2006; Djeri *et al*., 2010). This transformation method was initiated to aid yam preservation by dehydration of fresh tubers and solar drying and in turn reduces yam losses (Babajide *et al*., 2007). The processing of fresh yam tubers into chips is an activity that is carried out in Nigeria all round the year regardless of seasonal variations; a reason chiefly due to the fact that this food is on high demand by indigenes of the Southwestern and Northwestern Nigeria.

However, the chips produced through this traditional method have been reported to be attacked and deteriorated by moulds especially *Aspergillus*, *Fusarium*, *Penicillium* and *Rhizopus* amongst many other fungi (Adeyanju and Ikotun, 1988; Bankole and Mabekoje, 2004; Gnonlonfin *et al*., 2008; Djeri *et al*., 2010). Fungal contamination of this food material induces deterioration which is seen as discoloration of chips, loss of vital nutrients and possible contamination with mycotoxins (Bankole and Adebanjo, 2003; Djeri *et al*., 2010). The major toxins reported to contaminate yam chips in Nigeria and Benin Republic are aflatoxins (Bassa *et al*., 2001; Bankole and Adebanjo, 2003; Bankole and Mabekoje, 2004; Mestres *et al*., 2004), a group of zootoxic secondary metabolites from *A. flavus*, *A. parasiticus* and *A. nomius*.

The *Aspergillus* section *Flavi* is a group of aflatoxigenic and non aflatoxigenic taxa consisting of *A. flavus* (L- and S- types), S_{BG} unnamed taxon, *A. parasiticus*, *A. nomius* and *A. tamarii* (Cotty, 1994; Cotty and Cardwell, 1999; Ehrlich *et al*., 2003, 2004). The *A. flavus* L-type has been reported to be the most distributed in Nigerian soils and grains, majority of which are atoxigenic (Atehnkeng *et al*., 2008; Donner *et al*., 2009). Conversely, the contamination rate of food commodities with aflatoxin in Nigeria is high (Akano and Atanda, 1990; Adebajo, 1993; Adebajo and Idowu, 1994; Bankole and Eseigbe, 1996; Udoh *et al*., 2000; Atehnkeng *et al*., 2008). We could not lay hands on any report on the distribution of *Aspergillus* section *Flavi* strains in dried yam chip from Nigeria or their potential for aflatoxin production. Gnonlonfin *et al*. (2008)

highlighted the need for testing the *A. flavus* isolates from cassava and yam chips from Benin Republic for their toxigenic potential in a bid to assess the microbiological quality of the chips from the toxigenic fungi standpoint. Therefore, in line with this suggestion and considering the lack of data on the distribution and toxigenicity of *Aspergillus* section *Flavi* in yam chips in Nigeria, we carried out this study to provide data to strengthen previous reports on the microbiological quality of yam chips. The aims were to assess yam chips from Ibadan, Nigeria, for the presence of toxigenic *Aspergillus* section *Flavi* strains and quantify the aflatoxin content in the chips.

2. Materials and methods

2.1 Samples

A total of 40 dried yam chips were purchased from four markets in Ibadan, Nigeria: Omiadio, Oje, Ojaoba and Bodija. Each sample of 1kg size was collected in a sterile *zip-lock* bag from three points of the marketer's bag or basket and transported to the Mycology laboratory of Botany and Microbiology department, University of Ibadan, Nigeria for further analysis. Samples were comminuted, kept at 4°C and analyzed within 72 hours. The dried yam chips were produced locally by the indigenes of Ibadan, the major yam chip producing region of Southwestern Nigeria, and sold within and outside the state to as far as 200km locations away. Information from the marketers confirmed that the samples collected from Omiadio and Oje markets have been produced up to 3 months (90 days) prior to time of purchase while samples from Ojaoba and Bodija were produced 7-10 days prior to sample collection time.

A control sample was prepared within the laboratory following the routine method adopted by the producers. This sample was used to determine the effect of storage duration on fungal contamination and aflatoxin accumulation in the samples. For the preparation of control yam chips, healthy yam tubers with no traces of yam disease symptoms were thoroughly washed in clean water to remove adhering soil and other undesirable materials from the yam and to dislodge fungal fragments (mycelia and spores) on the yam peels. The tubers were peeled and sliced into 5-10cm wide, 2-3cm thick-sized chips so as to hasten the process of sun drying. The sliced yams were rinsed in clean water, parboiled for 10minutes and drained after which chips were sun dried for 3 days. The dried chips were comminuted, packed in sterile *zip-lock* bags and analyzed immediately.

2.2 Mycological analysis

Contaminating fungi of the food material were recovered according to Samson *et al*. (1995b). Briefly, each 10g sub sample of the powdered chips was serially diluted in 9ml of 0.1% sterile peptone water after the preparation of a 1:10 (v/v) stock solution. A 0.1ml aliquot from the 10^{-3} dilution tube for each sample was plated onto acidified Potato dextrose agar (PDA) in triplicates. Inoculated plates were incubated at 31°C for 3 days. Colonies with typical green appearance resembling *Aspergillus* were transferred to 5/2 agar plates (5% V8 juice and 2% agar, pH 5.2) for identification and differentiation of members of the section *Flavi* group (Atehnkeng *et al*., 2008). The 5/2 plates were incubated at room temperature for 5 days. The identification of *Aspergillus* section *Flavi* was based on macro- and micro-characters of members according to Klich and Pitt (1988), Cotty (1989), Cotty and Cardwell (1999) and Ehlrich *et al*. (2007). Other aspergilli and common moulds were also identified following the descriptions and illustrations in Raper and Fennel (1965), Pitt (1979) and Samson *et al*. (1995a).

2.3 Aflatoxigenicity testing of isolates

The ability of the *Aspergillus* section *Flavi* strains to produce B- and G-aflatoxins was tested *in vitro* following the liquid fermentation method of Cotty (1997). A total of 210 strains comprising of 198 market strains and 12 strains from the control chips were tested. The strains from the control chips were not included in the calculated incidence of toxigenic *Aspergillus* section *Flavi* in this study since the chip was meant to serve the purpose earlier stated. Isolates were screened for aflatoxin production in Adye and Matales (A&M) medium (Matales and Adye, 1965) containing 22.4 mM urea as the sole nitrogen source (pH 4.7) (Cotty and Cardwell, 1999). Small fermentation tests were carried out on the strains in 15 ml vials containing 5 ml A&M medium. Each vial was seeded with 100µl conidia suspension of approximately 1×10^6 conidia/ml and incubated at 31°C for 5 days. After incubation, 3ml acetone was added to each vial, vortexed for 10secs and allowed to stand for 1 hour to allow fungal cells to lyse and extraction of aflatoxin from cells. The content of each vial was filtered over a piece of Whatman No. 1 filter paper and the filtrate diluted before separation alongside standard aflatoxin B_1, B_2, G_1 and G_2. Separation of aflatoxins was carried out on 20×10 pre-coated aluminum thin chromatographic (TLC) plates developed in diethyl ether–methanol–water (96:3:1). The presence or absence of aflatoxin types was scored by visualizing the dried plates under high intensity ultraviolet light (365 nm).

2.4 Quantification of aflatoxins in dried yam chips

A 25 g representative sample of each powdered yam chips sample was extracted with 80%

methanol in water (v/v) in a 250 ml Erlenmeyer flask. The mixture was shaken for 30 min in a roto-shaker and allowed to settle. 0.2ml of the upper layer was separated on a 20×20cm pre-coated TLC plate (silica gel 60, 20mm) alongside aflatoxin B_1, B_2, G_1 and G_2 standard of known concentration. The plates were developed as stated above. The presence or absence of aflatoxin types was scored by visualizing the dried plates under high intensity ultraviolet light (365 nm). Visual estimation of aflatoxin B_1 (AFB_1) was carried out by comparing the intensities of fluorescence of the sample spots with that of the standard AFB_1 spots.

2.5 Statistical analysis

The mean incidences for strain distribution and toxigenic strains were calculated untransformed and values at $P<0.05$ were considered statistically significant. The differences between means for fungal incidence and proportion of toxigenic to atoxigenic strains was separated by the Duncan's multiple range test (DMRT) at $\alpha=0.05$. The relationships between the fungal incidence properties and aflatoxin content as well storage duration were assessed by correlation analysis.

3. Results

3.1 Incidence of common moulds and *Aspergillus* section *Flavi* species

All the yam chip samples used in this study were contaminated by moulds. Four fungal genera were identified: *Aspergillus*, *Fusarium*, *Neurospora* and *Penicillium*; in varying proportions from the dried yam chip samples across the four markets. *Aspergillus* species were the most isolated fungi in all samples (>56% incidence), having a significantly ($P<0.05$) higher incidence than all other identified fungal genera.

A total of 234 isolates belonging to *Aspergillus* section *Flavi* was recovered from 92.5% (37/40) of the yam chip samples collected from the markets in Ibadan and 66.7% of the control chips. The *A. flavus* L-strain was the most isolated taxon with significantly ($P<0.05$) higher incidence (>60%) than all other taxa within markets and control samples (Table 1). No significant difference ($P>0.05$) was observed in the incidence of *A. flavus* L-type across markets and control samples. The mean incidence of *A. tamarii* was not significantly higher ($P>0.05$) than the S_{BG} strain and *A. parasiticus*. S_{BG} strain and *A. parasiticus* were isolated only from Oje and Omiadio chips.

Table 1: Incidence of major taxa of *Aspergillus* section *Flavi* in dried yam chips collected from markets in Ibadan, Nigeria.

Samples	% contamination	Number isolated	% *A. flavus*	% S_{BG} strain	% *A. parasiticus*	% *A. tamarii*
Ojaoba	80.0	48	85.4[a]	0.0	0.0	14.6[b]
Oje	100.0	60	75.0[a]	4.2[a]	3.3[a]	17.5[b]
Bodija	90.0	54	62.0[a]	0.0	0.0	38.0[a]
Omiadio	100.0	60	82.5[a]	0.8[b]	3.3[a]	13.3[b]
Control	66.7	12	79.2[a]	0.0	0.0	20.8[b]
Total	90.7	234	---	---	---	---
[*]Mean (%)	---	---	76.3	1.3	1.7	20.7

[*]Mean percentage incidence of each major taxa of *Aspergillus* section *Flavi*
Means with same alphabet in a column are not significantly different ($P>0.05$).

3.2 Incidence of aflatoxigenic and non aflatoxigenic *A. flavus*

The aflatoxigenicity test for the *Aspergillus* section *Flavi* isolates showed that all toxigenic *A. flavus* strains produced only B-aflatoxins, thereby confirming their taxonomy as *A. flavus* L-type. The S-type strains and *A. parasiticus* produced both B- and G-aflatoxins. Since no S-type produced only B-aflatoxins, the strains were confirmed as S_{BG} unnamed taxon. All *A. tamarii* produced no aflatoxin while the incidence of toxigenic strains was highest (100%) in S_{BG} strains and *A. parasiticus*. The incidence of toxigenic *A. flavus* L-type ranged from 13.4% – 56.7% across the markets with a higher significant ($P<0.05$) mean incidence of atoxigenic *A. flavus* to toxigenic *A. flavus* (Table 2). Only 8.3% (1/12) of the *A. flavus* strains from the control chips were toxigenic for B-aflatoxins (data not shown).

The relative proportion of toxigenic to atoxigenic strains of *A. flavus* varied across markets with significant differences ($P<0.05$) in the atoxigenic incidence over toxigenic in all markets except Omiadio. Conversely, there was no significant difference ($P>0.05$) among toxigenic strains across markets, the atoxigenics across markets and overall relative proportion of toxigenic to atoxigenic strains (Table 3).

Table 2: Incidence of toxigenic taxa of *Aspergillus* section *Flavi* isolated from dried yam chips in Ibadan, Nigeria.

Samples	Number tested	% Toxigenic strains			
		% *A. flavus*	% S_{BG} strain	% *A. parasiticus*	% *A. tamarii*
Ojaoba	48	13.4	---	---	0.0
Oje	50	40.0	100.0	100.0	0.0
Bodija	50	56.7	---	---	0.0
Omiadio	50	50.6	100.0	100.0	0.0
% Toxigenic	---	39.3*	100.0	100.0	0.0
% Atoxigenic	---	60.7*	0.0	0.0	100.0

*Mean percentage proportion of toxigenic to atoxigenic *A. flavus* are significantly different (P<0.05).

Table 3: Mean incidence (%) of toxigenic and atoxigenic *Aspergillus* section *Flavi* isolated from yam chips in markets in Ibadan

Incidence (%)	% incidence by markets				Overall %	
	Ojaoba	Oje	Bodija	Omiadio	[a]Tox	[b]Atox
Toxigenic	11.5	37.0	38.0	45.0*	33.1[c]	---
Atoxigenic	88.5	63.0	62.0	55.0*	---	66.9[c]

[a]Tox: Toxigenic, overall % incidence of toxigenic strains in all yam chip samples across markets
[b]Atox: Atoxigenic, overall % incidence of atoxigenic strains in all yam chip samples across markets
[c]Overall % toxigenic to atoxigenic strains are not significantly different (P>0.05).
*Mean percentage values in a column are not significantly different (P>0.05).

3.3 Aflatoxin content in yam chips

B- and G-aflatoxins were detected in the chip samples obtained from the markets. All aflatoxin types (B_1, B_2, G_1 and G_2) were detected only in samples from Omiadio markets while the B-aflatoxins and at least one of the G types occurred in samples from all markets excluding Bodija (data not shown). The mean AFB_1 concentration was highest (190µg/kg) in samples from Oje market (Table 4). On the other hand, samples from Omiadio market were contaminated had the highest mean level of AFG_1 (141µg/kg). The overall incidence for AFB_1 contamination was 97.5%. The control yam chips were contaminated with only B-aflatoxins up to 2µg/kg.

Table 4: Summary of aflatoxin B_1 analysis in dried yam chips collected from markets in Ibadan, Nigeria.

Markets	Number analyzed	% positive samples	[a]Range of AFB_1	Mean AFB_1	[b]% samples above MAL
Ojaoba	10	90	14 – 21	17	20
Oje	10	100	169 – 216	190	100
Bodija	10	100	11 – 27	19	50
Omiadio	10	100	141 – 196	180	100
Total	40	97.5	---	---	67.5

[a]Range values (µg/kg) of aflatoxin B_1 (AFB_1) in samples from markets were approximated to nearest whole number
[b]% samples indicate samples contaminated above maximum acceptable limit (MAL) of 20 µg/kg.

When considering EC regulation for AFB_1 in foods, only 2/10 of the samples from Ojaoba market violated the set limit (20µg/kg). Conversely, all samples from Omiadio and Oje markets were contaminated with aflatoxin B_1 far above the maximum acceptable limits (MAL) (Table 4). On the overall, about 67% of the chip samples analyzed in this study violated EC's regulation.

The relationship between the incidence of *Aspergillus* section *Flavi* and aflatoxin concentration showed a positive correlation (r=0.63) as well as the relationship between the relative incidence of toxigenic *A. flavus* strains in the chip samples and aflatoxin concentration (r=0.66). There was a positive correlation (r=0.65) for the influence of storage duration on the incidence of *Aspergillus* section *Flavi* albeit a higher

positive correlation (r=0.99) was evident for the relationship between storage duration and aflatoxin accumulation (concentration) in the samples.

4. Discussion

An array of fungi belonging to four identified genera: *Aspergillus*, *Fusarium*, *Neurospora* and *Penicillium*, were isolated from the chips although other unidentified isolates contaminated the chips in very low quantities. This is in line with reports from similar studies in Nigeria, Benin Republic and Togo (Adeyanju and Ikotun, 1988; Bankole and Mabekoje, 2004; Gnonlonfin *et al*., 2008; Djeri *et al.*, 2010; Makun *et al.*, 2010). The 100% fungal contamination level of the chips and high incidence of contaminating fungi especially *Aspergillus* and *Fusarium* in this study reveal the pitfalls in the processing (especially at the washing and sun drying stages) and storage of this food material (Djeri *et al*., 2010). However, the very low incidence of fungi and number of *Aspergillus* section *Flavi* isolated from the control chips give us a direct clue to the contribution of storage duration on fungal incidence as well as toxin accumulation both of which were positively correlated in this study. It was observed that the chip samples from markets were not well stored after processing prior to sale. The samples were stored in humid corners in baskets, jute bags or wooden pots and were prone to attack by insects. Also when exhibited for sale in markets, they were exposed to the environment such that spores and fungal propagules in air arising from the numerous activities in the market would settle on them and finding a suitable niche, begin proliferation leading to deterioration of chips and subsequent toxin liberation. Amusa *et al*. (2003) and Cardwell and Henry (2005) stated clearly, the role of poor storage and insect infestation on fungal invasion, growth and subsequent metabolite liberation different food materials.

The contamination levels of *Aspergillus* section *Flavi* especially *A. flavus* (>75% mean incidence) exceeded the tolerable limit as specified by the International Commission on Microbiological Specification for Food (ICMSF). This shows the low quality of chips available for human consumption in this region of Nigeria and the possible health risk involved since it has been noted that consumption of fungal spores and their mycotoxins are a great risk to humans and animals (Peraica *et al.*, 1999; Bryden, 2007). A similar data for the occurrence and violation *Aspergillus* was reported by Gnonlonfin *et al.* (2008) in Benin Republic.

The significantly (P<0.05) high incidence of *A. flavus* L-type in the chips as compared to other taxa may be linked to the corresponding high occurrence levels of *Aspergillus* section *Flavi* in Nigerian soils and food materials (Atehnkeng *et al.*, 2008; Donner *et al.*, 2009). This is similar to reports from other parts of the globe including West Africa (Horn and Dorner, 1998, 1999; Cardwell and Cotty, 2002). Since we could not lay hands on any report for the occurrence of the different *Aspergillus* section *Flavi* taxa on dried yam chips, our study provides the first available data for the occurrence of *Aspergillus* section *Flavi* taxa in dried yam chips. The occurrence of the S_{BG} unnamed taxon, though at very low incidence levels (<2%), and *A. parasiticus* in the samples raises much concern especially towards aflatoxin accumulation in the samples since all strains of both taxa are aflatoxigenic and produce very high amounts of both B- and G-aflatoxins (Cotty and Cardwell, 1999; Cardwell and Cotty, 2002; Atehnkeng *et al.*, 2008; Donner *et al.*, 2009). In addition, the occurrence of S_{BG} strain in these samples from Southwestern Nigeria is noteworthy since Ibadan, our study location, falls under the Derived Savannah (DS) agro-ecozone of Nigeria. Previous reports of the occurrence of the S_{BG} unnamed taxon in Nigeria and other parts of West Africa has shown its preference and clustering within the drier Northern parts (Cardwell and Cotty, 2002; Atehnkeng *et al.*, 2008; Donner *et al.*, 2009). However, Donner *et al*. (2009) reported its very low incidence within maize soils from several locations within the DS agro-eco-zone.

The proportion of atoxigenic to toxigenic *A. flavus* occurring in the chips varied from location to location and was generally on the higher side for the atoxigenics except for one location, Omiadio market. This is similar to previous reports of the distribution of atoxigenic and toxigenic *A. flavus* in Nigerian soils and food (Atenhnkeng *et al*., 2008; Donner *et al*., 2009) and in Argentina and Iran where toxigenic species are <30% (Vaamonde *et al.*, 2003; Razzaghi-Abyaneh *et al.*, 2006). However, reports from other parts of the globe such as in the United States indicate a higher proportion of aflatoxigenic to non aflatoxigenic *A. flavus* (Cotty, 1997; Garber and Cotty, 1997; Horn and Dorner, 1999).

The high concentrations of B- and G-aflatoxins in the market chips indicate the extent of poor storage conditions to which the chips are exposed and the associated health risks posed to the consumers, many of which are not aware of the health implications of ingesting mycotoxins. The mean AFB_1 contamination level and concentration values recorded in this study are slightly higher than what was reported in previous studies on aflatoxin contamination of yam chips in Ibadan, Nigeria by Bakonle and Adebanjo (2003) and Bankole and Mabekoje (2004), and in Benin Republic by Bassa *et al*. (2001) and Mestres *et al*. (2004). However, our report was a direct contrast to the findings of Gnonlonfin *et al.* (2008) who did not detect any aflatoxins or fumonisin B_1 in cassava and yam chip

samples from Benin Republic. Meteorological data obtained for Nigeria indicate that the year of our study was hotter and drier than the warmer and cooler years during which previous researchers carried out their analysis. Processing conditions and length of storage of chips samples before analysis may have also contributed a lot to the disparity in AFB_1 concentration values obtained. Aflatoxin production has been reported to be influenced greatly in hotter and drier climates than in warm colder regions (Guo *et al*., 2005) and reduced drastically in a microenvironment by the presence of a wide array of competing fungi (Velluti *et al*., 2000), a data that was not really obvious in our study. It may then be suggested that other potential competitors were excluded from this substrate due to the prevailing climatic conditions (mean temperature, 33^{o}C; relative humidity, 16%).

Although the presence of fungi in a material does not necessarily denote toxin contamination, we observed positive correlations between fungal properties and aflatoxin accumulation as well as storage conditions (physical factors). From the correlation results, we may suggest that once the chips or a food material has been initially contaminated longer storage duration under convenient conditions for fungal proliferation will give room for higher aflatoxin accumulation. However, this is dependent on other factors such as presence of other fungal components which may cause high competition, and nature of food material (Horn and Wicklow, 1983; Velluti *et al*., 2000). This is in line with the reports of Bankole and Eseigbe (1996) who stated from their study that there was likely to be a correlation between the incidence of *A. flavus* and aflatoxin concentration in tiger nuts from Nigeria.

Considering the role of these contaminating fungi in food poisoning and the associated health problems coupled with the challenges posed by aflatoxin contamination in trade and economics (Bankole and Adebanjo, 2003; Bryden, 2007) we recommend that prompt attention be given to this widely consumed food material by the regulatory agencies regardless of its traditional background. The processors should pay great attention to the processing and storage conditions especially the drying environment, duration and proper packaging before sale. For the consumer, it is not necessary to stop or quit consumption of yam chips (and its products) due to fear of consuming fungal infested or aflatoxin contaminated yam chips; instead there is need for selective buying of fresh and crispy yam chips. Further work will be directed towards the multi-toxin analysis of the chips within Nigeria in order to provide more toxin data for legislation by regulatory agencies as well as searching for traditional herbs that may be used to mitigate fungal attack of the chips.

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