Infection of Termite Scarified Groundnuts by *Aspergillus* Section *flavi* and Contamination by Aflatoxin

Kwanashie A.J.[1]*, Alabi O.[1], Onu I.[1] and Inuwa H.M.[2]

[1] Department of Crop Protection, Faculty of Agriculture, Ahmadu Bello University, Zaria – Kaduna State

[2] Department of Biochemistry, Ahmadu Bello University, Zaria – Kaduna State

*Corresponding author

Summary

Groundnuts are invaded by members of *Aspergillus* section *Flavi* (*Aspergillus flavus, A. parasiticus, A. tamari, A. caelatus and A. alliaceus*) of which *A. flavus* and *A. parasiticus* sequester carcinogenic toxins known as aflatoxins. These aflatoxins producing fungi infect groundnut pods/seeds during maturation with the highest fungal population and aflatoxin contamination occurring in damaged seeds. One hundred and fifty six groundnut samples comprising termite scarified and uncompromised pods were collected from farmer's fields in Zamfara, Katsina and Kano states, Nigeria. Groundnut seeds were found to be infected by *A. flavus* as well as *A. niger*. The highest incidences of *A. flavus* and *A. niger* were seen to occur in damaged seeds obtained from Kano (47%/10%) and Katsina (10%/9%) states. The undamaged seeds had a lower incidence of the fungi in Kano (38%/0%) and Katsina (7%/7%) Aflatoxin B_1 concentrations between the scarified and undamaged seeds were statistically insignificant. Mean aflatoxin B_1 concentration in scarified seeds across the three states was higher (19.65 µg/kg) than in the undamaged seeds (4.21 µg/kg). Ninety two per cent of samples had aflatoxin B_1 concentrations below the 20 µg/kg regulatory limit in Nigeria. The colonization of groundnuts by soil inhabiting aflatoxigenic *A. flavus* is enhanced by termite damage to the pods. This leads to an increase in the synthesis and concentration of the carcinogenic aflatoxin B_1 in infected seeds.

Key words: Groundnuts, *Aspergillus flavus*, Termites, Scarification, Aflatoxin B_1

GROUNDNUT (*Arachis hypogaea* L.) is the sixth most important oil seed crop in the world. It contains 48-50% oil, 26-28% protein, 11-27 % carbohydrate, minerals and vitamin (15). Nigeria is the third highest producer of groundnut in the world with a production of 3,413,100 tons after China and India with yields of 16,481,700, 6,557,000 tons respectively in 2014 (7). In Nigeria, the crop is grown throughout the country except for the riverine and swampy areas. Groundnut is either cultivated sole or in mixtures with other crops like maize, sorghum, millet or cassava. The leading producing states are Niger, Kano, Jigawa, Zamfara, Kebbi, Sokoto, Katsina, Kaduna, Adamawa, Yobe, Borno, Taraba, Plateau, Nasarawa, Bauchi, and Gombe States (16). Groundnut has contributed immensely to the development of the Nigerian economy. From 1956 to 1967, groundnut products including cake and oil accounted for about 70% of total Nigeria export earnings, making it the country's most valuable single export crop ahead of other cash crops like cotton, oil palm, cocoa and rubber (9). Presently, it provides significant sources of cash through the sales of seeds, cakes, oil and haulms (24). Confectionary products such as snack nuts, sauce, flour, groundnut butter and cookies are made from high quality nuts of the crop. In Northern Nigeria, apart from being consumed whole, groundnuts are processed into or included as an ingredient in a wide range of other products which includes groundnut paste which is fried to obtain groundnut cake (*kuli kuli)*, salted groundnut (*gyada mai gishiri)*, a gruel or porridge made with millet and groundnut (*kunun gyada)*, groundnut candy *(kantun gyada)* and groundnut soup (*miyar gyada)* (15).

Groundnuts are invaded by members of *Aspergillus* section *Flavi* (*Aspergillus flavus, A. parasiticus, A. tamari, A. caelatus and A. alliaceus*) of which *A. flavus* and *A. parasiticus* are aflatoxigenic. Aflatoxins are naturally occurring toxins produced these fungi and there are several types (B1, B2, G1, and G2) produced, of which aflatoxin B1 is the most toxic (21). Soil is a source of primary inoculum for *Aspergillus flavus* and *A. parasiticus*, fungi that produce highly carcinogenic aflatoxins in groundnuts. Aflatoxins have been shown to lead to aflatoxicosis in man and animals (12) which leads to an increase in the incidence of human hepatocellular carcinoma by acting in consonance with hepatitis viruses (1). Aflatoxigenic fungi commonly invade groundnut seeds during maturation, and the highest concentrations of aflatoxins are found in damaged seeds. This damage could be caused by foraging activities of termites, millipedes and the scarabeid beetle larvae.

We hypothesise that scarification by termites of groundnut pods increases the surface area for infection by *A. flavus* and subsequent accumulation of aflatoxins in the seeds. The current study was conducted to provide a basis for a better understanding of the symbiosis between termite damage and *Aspergillus* sp. colonization of groundnuts as it affects aflatoxin contamination. Mature groundnut samples consisting of both termites scarified and undamaged pod were collected from farmer's fields across three groundnut growing states in the northwest of Nigeria (Kano, Katsina and Zamfara). *A. flavus* was isolated from the samples and the seeds were subjected to enzyme linked immunosorbent assay to determine concentrations of aflatoxin B_1.

MATERIALS AND METHODS

Sample collection

Groundnut pod samples were collected from termite infested farmer's fields Kano and Katsina state during the 2016 harvest season. Groundnut pods were then sorted into scarified and non-scarified portions, shelled and stored separately in paper bags at 4^oC for further analysis.

Fungi isolation and Identification

One hundred (100) seeds were surface sterilized in 1% sodium hypochlorite for three minutes and rinsed with distilled water three times. Seeds were then plated on Potato Dextrose Agar (supplemented with streptomycine) and isolates belonging to *Aspergillus* section Flavi were isolated. Sub-cultures were done to obtain pure cultures (3). After 5 days un-illuminated at room temperature (27^oC - 31°C), isolates were classified on the basis of colony characteristics and conidial morphology. One representative sample from each state so identified, was sent to the Centre for Agriculture and Bioscience International (CABI), Surrey, United Kingdom for confirmation.

Aflatoxin Quantification

A blender was used to crust to powder 100 g of groundnut kernels. Twenty grams of this powder was mixed in 100 ml 70% methanol (v/v- 70 ml absolute methanol in 30 ml distilled water) containing 0.5% KCl and the mixture was blended until the mixture was thoroughly homogenized. The extract was transferred to a conical flask, sealed with parafilm and put on a mechanical shaker for 30 minutes at 300 rpm. The mixture was then filtered through a Whatman No. 4 filter paper, and the filtrate was stored at 4°C till needed for analysis. The filtrate was then diluted as appropriate, loaded alongside standards of aflatoxin B_1 and read off an Enzyme Linked Immunosorbent Assay (ELISA) reader. To estimate lower levels of AFB1 (< 10 µg/kg), prior to ELISA a simple liquid-liquid clean up and concentration procedure was adopted. Twenty milliliter of methanol extract, 10 ml distilled water and 20 ml chloroform were mixed in a separating funnel. After vigorous shaking for one minute, collect the lower chloroform layer was collected and evaporated to near dryness in water bath at 60^{o}C. To the residue 4 ml PBS-Tween containing 7% methanol was added and used for analysis by ELISA.

Data analysis

Analyses were performed with SAS (version 9.1.3, SAS Institute Inc., Cary, NC). Analysis of variance was performed on all data with the general linear model (GLM). Least significant difference (LSD) test was performed to compare treatment means at the 5% level.

RESULTS

Isolation and Identification of Fungi

Aspergillus flavus and *Aspergillus niger* were the twospecies isolated and identified from collected samples (Tables 1 and 2). Termite scarified samples showed a higher percentage occurrence of these fungi as compared to the non-scarified samples. Isolates presented with the characteristic dark green colony colouration with the reverse side being hyaline. Microscopic examination revealed the globose, ellipsoid and slightly spherical conidia.

Aflatoxin Quantification

Statistical analysis of Aflatoxin B_1 levels show no significant difference between the termite scarified groundnut pods/seeds and non-scarified obtained from Kano state (Table 3). There also presents no statistical significance in aflatoxin B_1 quantity between the local government areas sampled. There are however clear differences in the mean values between scarified (30.41 µg/kg) and non-scarified (5.76 µg/kg) seeds. Aflatoxin B_1 mean concentrations also differed across the local government areas considered with Dawakin Tofa having the highest (30.60 µg/kg) followed by Dawakin Kudu (12.72 µg/kg) and Minjibir (7.95 µg/kg) having the least (Table 3).

Aflatoxin B_1 levels, under statistical scrutiny (Table 4), show no significant difference between the termite scarified groundnut pods/seeds and un-scarified pods/seeds obtained from Katsina state within and across the two-year sample period. There is no statistical significance in aflatoxin B1 quantity between the local government areas sampled. There are however clear differences in the mean values between scarified (6.05 µg/kg) and non-scarified (0.67µg/kg) seeds. Aflatoxin B_1 mean concentrations also differed across the local government areas considered with Rimi having the highest (6.39µg/kg) followed by Batagarawa (3.44 µg/kg) and Rimi (0.26 µg/kg) having the least (Table 4).

Higher percentage of the groundnut samples (95.2 %) assayed for aflatoxin contamination fell within the Nigerian permissible limit of 20 µg/kg in Katsina state. Kano state shows a higher percentage of samples (90 %) containing the toxin to be below the Nigeria limit. Katsina (82.7%) showed levels lower than the European Union's set limit of 4 µg/kg with Kano (32%) having the lowest percentage in that range. AFB_1 maximum and minimum varied between the states with Kano (438.3 µg/kg and 0.1 µg/kg) showing the highest, followed by Katsina (46.2 % and 0.0 µg/kg) (Table 5).

Table 1: Percentage incidence of *Aspergillus* species associated with groundnut seed samples from Kano and Katsina states.

S/N	State	Isolated Fungi			
		A. flavus (%)		*A. niger* (%)	
		SC	NSC	SC	NSC
1	Kano	47	38	10	0
2	Katsina	10	7	9	7

SC = Scarified seeds NSC = Non-scarified seeds

Table 2: Identification of isolates by Centre for Agriculture and Bioscience International (CABI), Surrey, UK

S/N	Sample Number	IMI Number	Identification and Comments
1	K1	*506178	*Aspergillus flavus* Identification was made by macroscopic and microscopic analysis of subcultures prepared on diagnostic media. This isolate was identified as *Aspergillus flavus*. Morphology of colonies and sporulating material matched species descriptions provided in published taxonomic keys (e.g. Klich M. (2002) Identification of Common *Aspergillus* Species pp 46-47. Central Bureau voor Schimmel cultures,

			Utrecht, Netherlands).
2	K2	*506179	*Aspergillus flavus* Identification was made by macroscopic and microscopic analysis of subcultures prepared on diagnostic media. This isolate was identified as *Aspergillus flavus*. Morphology of colonies and sporulating material matched species descriptions provided in published taxonomic keys (e.g. Klich M. (2002) Identification of Common *Aspergillus* Species pp 46-47. Central Bureau voor Schimmel cultures, Utrecht, Netherlands).
3	K3	*506180	*Aspergillus flavus* Identification was made by macroscopic and microscopic analysis of subcultures prepared on diagnostic media. This isolate was identified as *Aspergillus flavus*. Morphology of colonies and sporulating material matched species descriptions provided in published taxonomic keys (e.g. Klich M. (2002) Identification of Common *Aspergillus* Species pp 46-47. Central Bureau voor Schimmel cultures, Utrecht, Netherlands).

Representative samples from each state were positively identified as *Aspergillus flavus*.

NB: K1 – Kano isolate; K2 – Katsina isolate; K3 – Zamfara isolate

Table 3: Aflatoxin B_1 quantification of groundnut samples collected in Kano State

Treatment	Aflatoxin Content (µg/kg)
Damage Level	
Scarified	30.41a
Un-scarified	5.76a
SE±	14.70
State (Kano)	
Dawakin Kudu	12.72a
Dawakin Tofa	30.60a
Minjibir	7.95a
SE±	18.0

SE = standard error. Means followed by the same letter(s) within the same column are not statistically different at 5% level of probability.

Table 4: Aflatoxin B_1 quantification of groundnut samples collected in Katsina State

Treatment	Aflatoxin Content (µg/kg)
Damage Level	
Scarified	6.05a
Un-scarified	0.67b
SE±	1.7
State (Katsina)	
Batagarawa	3.44a
Charanchi	0.26a
Rimi	6.39a
SE±	2.08

SE = standard error

Means followed by the same letter(s) within the same column are not statistically different at 5% level of probability.

Table 5: Distribution of aflatoxin contamination (AFB_1) on groundnut in three North-western states of Nigeria

State	Number of Samples	Number of samples with aflatoxin B_1 in range (µg/kg)								Max AFB_1	Min AFB1
		≤ 4 (EU)	%	4-15 (EU, Australia, Canada)	%	15-20 (Nigeria, USA)	%	>20	%		
Kano	50	16	32	25	50	4	8	5	10	438.3	0.1
Katsina	52	43	82.7	4	7.7	3	5.8	2	3.8	46.2	0.0

DISCUSSION

The incidence of *Aspergillus flavus* on groundnuts obtained from Kano and Katsina states show that the pre-harvest colonization of groundnuts with the fungus is common. *A. flavus* is a cosmopolitan filamentous, saprophytic fungus that frequently infects oil-rich seeds of various crop species during pre- and post-harvest periods (5) Although *A. flavus* is often described as a storage mold, it has been widely accepted that the infection with this fungus occurs in the field (20). Experiments showed a higher incidence of *A. flavus* on seeds obtained from termite damaged (scarified) groundnut pods with Kano state showing a higher incidence across the two damage levels (scarified and non-scarified) Research has shown that wounding of pods/seeds leads to an increase in infection by *A. flavus* (10). The lower incidences of colonization in viable non-scarified (undamaged pods/seeds) gives credence to earlier research showing the importance of damage in the systematic invasion by soil fungal fauna (2). Pre-harvest contamination of groundnuts with aflatoxins produced by colonising *A. flavus* is a global problem which pertains to human and animal health and food safety. The fungus is known to sequester mycotoxins such as the highly toxic carcinogens known as aflatoxins (B_1, B_2, G_1 and G_2) cyclopiazonic acid and aflatrem which have been implicated in both acute and chronic toxicity (aflatoxicosis) in both animals and humans (14). Aflatoxicosis in humans presents as acute liver damage and cirrhosis, tumorigenesis and teratogenic effects (13).

The detection of Aflatoxin B_1 indicates the toxigenicity of the *Aspergillus flavus* isolated from groundnut pods irrespective of their damage levels. This assertion finds literary concurrence with Fakruddin *et al*., (6) who characterized *A.flavus* isolated from groundnuts as aflatoxigenic producing aflatoxin B_1, Nyirahakizimana *et al*., (17) who reported the occurrence of *A.flavus* and the subsequent aflatoxin contamination in raw and roasted groundnuts and Ouattara – Sourabie *et al*., (18) who examined and showed the aflatoxigenic potential of *A. flavus* isolated from groundnut seeds using fluorescence and high performance liquid chromatography methods. *A. flavus* is an opportunistic fungus which produces aflatoxin as a secondary metabolite in the seeds of groundnuts both before and after harvest (11).

Concentration of aflatoxin B_1 determined using enzyme linked immunosorbent assay revealed higher concentrations of detected aflatoxin in seeds from scarified pods than seeds from non-scarified pods across the two states sampled. Statistical analysis shows no significant difference in mean concentration values but direct comparison of means show a marked difference in toxin concentrations between scarified and non-scarified seeds. Secondary metabolites such as tannins, waxes, amino compounds and structural features in the groundnut seed coat have been implicated in resistance to invasion by *A. flavus* and *A. parasiticus* (19, 25). Loss or reduction of these structural and biochemical structures due to damage caused by termites or other soil borne arthropods or nematodes lead to the higher incidences of *A. flavus* and concurrent concentrations of aflatoxin B_1. Increased colonization of damaged groundnuts by *A. flavus* has been documented (23, 10). This research has shown an increase in colonization of damaged groundnut seeds by aflatoxigenic *A. flavus*. The differential quantities of aflatoxin B_1 between scarified and non-scarified seeds correlates with the recorded increased infection by *A. flavus* in these seeds.

Ninety per cent of samples assayed from Kano state and 96.2 % of samples assayed from Katsina state showed concentrations of aflatoxin B_1 less than or equal to 20 µg/kg. These satisfy the Nigerian regulatory limit of 20 µg/kg (22). Due to the toxic and carcinogenic properties of aflatoxins, only extremely low levels of aflatoxins in foods and feeds is allowed (8). Many countries have enforced or recommended aflatoxin permissible limits for food produce/products which range from zero detectable to 50 µg/kg (13) Countries have multiple limits depending on the utilization of the produce, the stringent limits pertaining to human consumption and exports, and the highest to commercial products. These restrictions result in a loss in trade revenues arising from increased cost of meeting the standards – including cost of testing, rejection of shipments and even eventual loss of admissibility into foreign markets (4).

Good agricultural practices at both pre-and post-harvest stages of groundnut including storage are available to prevent aflatoxin build up in groundnut. Management of termites and other soil borne arthropods in groundnut fields will reduce the incidence of *A. flavus* and aflatoxin contamination.

CONCLUSION

Aspergillus flavus colonizes groundnut seeds with increased populations isolated from seeds that were damaged by termites. The *A. flavus* specie isolated was shown to be aflatoxigenic as evident in the detection of aflatoxin B_1 in all groundnut samples analysed. The damage of seeds not only leads to an increase in population densities of *A. flavus* but also the concurrent increase in aflatoxin $B_{1.}$ Aflatoxin B_1 concentrations levels in groundnut seeds at or immediately after harvest is lower than the regulatory standards of Nigeria of 20 µg/kg. Although these levels are permissible, increase in concentrations may occur in storage if adequate control of the fungus is not ensured.

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