STUDIES ON FUNGI AND MYCOTOXINS ASSOCIATED WITH DECAYED TIGERNUTS (*CYPERUS ESCULENTUS* L.) FROM KEFFI LOCAL GOVERNMENT AREA, NASARAWA STATE, NIGERIA

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ABSTRACT

A study was carried out on Fungi associated with decayed tigernut and their mycotoxin producing ability. Four (4) locations namely Angwan Lamba, Angwan Jaba, Keffi Market and Angwan Kaje were covered. Of the 120 tubers collected during the 2 visits, 53.34% had various fungi isolates. Identified fungi species were: Aspergillus, flavus, Rhizopus oryzae, Aspergillus niger and Fusarium solani; whose occurrence were 29.69%, 14.06%, 28.13% and 28.13% respectively. Angwan Kaje had the highest incidence of fungal species (10.00%). Chi-square analysis of the data obtained showed that there was no significant difference (P<0.05) in the incidence of different isolates in relation to different locations in Keffi Local Government Area. Results from the mycotoxin analysis showed presence of aflatoxin B1. Concious efforts by Keffi L.G.A authority should be geared towards discouraging people from consuming infected tiger nut tubers.

Keywords*:* Fungi, decay, mycotoxins, Tigernut tubers, Keffi L.G.A

INTRODUCTION

Tigernut (*Cyperus esculentus* L.), belongs to the family Cyperaceae (Uva *et al*., 1997) is a tuberous plant consumed widely in Nigeria and some parts of West and East Africa (Abaejoh *et al*., 2006). The tubers are eaten like nuts or pounded into cakes and served at the end of as desserts. Eteshola and Oreadu (1996) reported that Tigernut oil is of high nutritional value and can be used in food products. It has been estimated that human dietary lipids requirement would be satisfied by eating 150 - 200g tubers per day. Tigernut tubers are known to have an excellent nutritional qualities with a fat composition similar to Olives and a rich mineral content especially phosphorus and potassium (Eteshola and Oreadu, 1996). Tigernut tubers also contain myristic acid as the predominant unsaturated acid.

Tigernut tubers are also used as a feeds for livestock as reported by Bamgbose *et al*. (1997). Tigernuts can be used as supplements for feeds, which may be deficient in lysine. This supplementation process can be concentrated for man and livestock, since tubers can be grown fairly well on poor soil (Hayes, 1981).

Decays and deteriorations occur on tigernuts caused by *Aspergillus niger, Aspergillus flavus* and *Fusarium solani* (Bhat, 1988). These fungi produce mycotoxins which are carcinogenic and mutagenic (World Health Organisation, 1979). Mycotoxins are low molecular weight toxic compounds produced by certain toxigenic strains of a variety of filamentous fungi belonging to the genera *Aapergillus,Penicillium, Alternaria* and *Fusarium.* Of these genera, the first three are the major contributor of mycotoxin production in grain and fruit (Drush and Ragab, 2003). Following harvest, the defense mechanism of grains and oil seeds against fungal invasion decreases (Annor *et al*., 2004) and therefore, fungal population may increase and subsequently result in the production of mycotoxins during drying processing and storage (Hell *et al*., 2009). Mould growth and mycotoxin production in grains and oil seeds and other agro-allied commodities are very important to producers, processors and consumers of such as groundnut (Akano and Atanda, 1989); melon seed (Bankole and Jola, 20004); and some fungal species have been reported to have deteriorated castor oil seed (Negedu, 2009) and tigernut (Onovo and Ogaraku, 2007). This study therefore is aimed at investigating the fungi associated with deteriorated tigernut and mycotoxin producing ability of these fungi obtained from Keffi Local Government Area, Nasarawa State, Nigeria.

MATERIALS AND METHODS

Collection of samples

Deteriorated tigernut samples were cultured and in Plant Science and Biotechnology Unit Laboratory, Department of Biological Sciences, Nasarawa State University, Keffi. Keffi lies between latitude 8º50N and longitudes 7º52E. Mycotoxin analysis was conducted at Biological Sciences Department, University of Agriculture, Markudi, Benue State, located on latitude 6º35N and longitude 5º35E (Akwa *et al*., 2007). The deteriorated samples were collected from locations randomly selected in Keffi Local Government Area. These locations include; Angwan Lambu Angwan Jaba, Keffi Market and Angwan Kaje.

Tigernut sellers were visited twice (2) for the collection of deteriorated tigernut tubers in June and July, 2012. During the first visit, fifteen (15) deteriorated tubers were aseptically collected from one seller per location in a sterile plastic; hence for the four (4) locations, a total of 60 tubers were obtained. For july, another sixty (60) tubers were collected from the same seller per location making a total of 120 deteriorated tubers. Hence, there were thirty (30) sample replicates per location. For the four locations there were 120 deteriorated tigernut samples for culturing.

Data were obtained from the proportions of tubers infected with fungal species. Data obtained from the survey were subjected to chi-square for analysis.

Fungal Isolation and Identification.

The isolation technique for fungi was described by Dongmo and Oyeyiola (2006). Decayed tubers were rinsed in water, surfaced sterilized with 70% ethanol, infected tissue were picked with a flame-sterilized

forceps and plated on Sabouraud agar medium obtained from sigma Aldrich Co. Ltd and incubated (model – DHG-9053A) at 30^0C for 7 days. For fungal identification, photomicrography of the isolates was obtained from the cultures. Fungal structures observed were compared with micrograph of Samson *et al*. (1984). Pure cultures were maintained in a refrigerator at – 4^0C to be used for artificial inoculation.

Artificial Inoculation

One disc (5mm diameter) of mycelia and agar of the inocula of *Aspergillus flavus*, *Rhizopus oryzae*, *Aspergillus niger*, and *Fusarium solani* were aseptically introduced separately using spatula into four 25ml McCarthney bottles containing 50g of tigernut. (each fungus into one bottle). One bottle each of 25ml McCarthney bottle containing 50g of tigernut was left as control. Each bottle containing autoclaved, inoculated tubers was left at room temperature (30^0C) for four (4) months (Gbodi *et al*., 1986).

Mycotoxin Extraction from inoculated samples

Hammer mill pulverized sample of inoculated tubers was weighed into 500ml Erlenmeyer flask, 25ml methylene chloride added, flask was shaken for 30mins on a rotary shaker and the contents filtered through 3cm filter paper on a Buchner funnel. Filtrate was collected. Fifty milliliters (50ml) aliquots were placed in four separate 100ml Erlenmeyer flasks and evaporated to dryness in water bath at 60^0C. For assay of mycotoxins, dried extract was re-dissolved in 20ml benzene: acetonitrite (98:2 v/v) and shaken thoroughly to ensure complete dissolution of toxins (Gbodi *et al*., 1986).

Characterisation of the Mycotoxins using Thin Layer Chromatography

Pre-coated chromatographic plates (20cm x 20cm silica gel) were activated at 110ºC for thirty (30) minutes before use and prior to spotting; the prepared stock solutions of the standard toxins were thoroughly shaken twice. On the chromatographic plate, at 2cm from the bottom 5ml of the dissolved extracts were spotted using a 10µl glass syringe (Unimetrics, Shore wood, Illinois, 60436, USA). Beside the sample spots, on the same plate, 5 µl of the standard mycotoxins (Aflatoxin B1 and B2, Ochratoxin) were spotted at 2cm from the bottom of the plate. Four sample extracts were spotted each time (Gbodi *et al*., 1986).

Development of Chromatographic plate

Appropriate developing solvent (toluene: ethylacetate: formic acid (60:40:1 v/v) was shaken

and poured into a Thin Layer Chromatographic tank (TLC) to about 1cm depth. Each plate was developed by standing in chromatographic tank containing the developing solvent and developed until the solvent front was at least 9cm from the tip of the plate. Solvent front was immediately marked as distance moved by the mobile phase and recorded (Gbodi *et al*., 1986).

Detection of Mycotoxins in the Sample Extracts

Development plate were dried and examined visually under ultra violet (UV) light and fluorescent intensities of the samples spots were compared and recorded together with the aliquot volumes that were spotted. The retention factor (Rf) of the standard and that of sample spots were calculated as $\frac{\text{"distance moved by the solutes"}}{\text{"distant moved by the solvent front"}}$

Mycotoxin aspects, constituents in sample extracts was detected by matching fluorescent colour, intensities and the retention factor (Rf) value of samples spots with the standard spots. Sample spots whose fluorescent intensities, retention factor (Rf) values matched those of the standard were presumptively considered positive and used for confirmation (Gbodi *et al*., 1986).

Confirmation of Mycotoxin Presence

The TLC plates that were presumptively considered positive were sprayed firstly with mycotoxin extract dried and viewed again at 365nm to confirm the presence of aflatoxin (a blue fluorescence colour would confirm aflatoxin).

Presence of Ochratoxin was confirmed spraying alcoholic aluminum chrolide (20g / 100ml alcohol) and exposure of the plate to NH_2 vapour and viewed at 254nm. Change in colour from blue green to bright blue fluorescence confirms ochratoxin (Gbodi *et al*., 1986).

RESULTS

Of the one hundred and twenty (120) tubers decayed nut were collected and sampled, out of which 64 tubers had fungal isolates, organisms while 56 tubers were without fungal isolates (Table 1). Angwan Kaje had the highest incidence of fungal species (15.00%) while Keffi market had the least incidence (10.00%).

Fungal species isolated and identified from deteriorated tigernut samples were *Aspergillus flavus, Rhizopus oryzae*, *Aspergillus niger* and *Fusarium solani*. The percentage distribution of these fungi was 29.69, 14.06, 28.13, and 28.13 respectively (Table 2).

Chi-square analysis of the data obtained showed that there was no significant difference ($P<0.05$) in the incidence of different isolates in relation to different locations in Keffi Local Government Area (Table 3). The result of the multi-thin layer chromatographic analysis of fungal infected tubers compared with that of the standard mycotoxins is shown in Table 4. Aflatoxin B1 was produced by both *Aspergillus flavus* and *Aspergillus niger* with the same retention factor 0.71 while *Rhizopus orysae* and *Fusarium solani* produced no mycotoxin.

DISCUSSION

Fungi responsible for the deterioration of tigernut tubers in Keffi Local Government Area are *Aspergillus flavus, Rhizopus oryzae, Aspergillus niger* and *Fusarium solani*. Photomicrography of the isolates was obtained and

Table 1: Incidence of Fungal species in tigernut tuber examined from different locations in Keffi Local Government Area

Location	Total No. of tubers Examined	No. with fungal spp.	No. without fungal spp
Angwan Lambu	30	17 (14.17)*	13 (10.83)*
Angwan Jaba	30	17 (14.17)*	13 (10.83)*
Keffi Market	30	12 (10.00)*	18 (15.00)*
Angwan Kaje	30	18 (15.00)*	12 (10.00)*
Total	120	64 (53.34)*	56 (46.66)*

* Numbers in parenthesis are in percentages

Table 2: Distribution of fungi isolated from tigernut sourced from different locations in Keffi Local Government Area

Location	*Aspergillus flavus*	*Rhizopus oryzae*	*Aspergillus niger*	*Fusarium solani*	Freq. of occurrence
Angwan Lambu	04	02	08	03	17
Angwan Jaba	05	01	05	06	17
Keffi Market	03	03	04	02	12
Angwan Kaje	07	03	01	07	18
Total	19	09	18	18	64

Table 3: Chi-Square on the Relationship between Fungal Isolates and Different locations in Keffi Local Government Area

Location	No. with fungal spp.	No without fungal spp.	Total
Angwan Lambu	17 (16.00)*	13 (14.00)*	30
Angwan Jaba	17 (16.00)*	13 (14.00)*	30
Keffi Market	12 (16.00)*	18 (14.00)*	30
Angwan Kaje	18 (16.00)*	12 (14.00)*	30
Total	64	56	120

Numbers in parenthesis are expected values

H_o: There was no significant difference ($P<0.05$) in the incidence of different isolates in relation to location in Keffi.

F.Cal. 7.81 < Tab 8.84, therefore we accept H_o

fungal structures observed were compared with micrograph of Samson *et al*., (1984). Most of these species of fungi have also been reported by Abaejoh *et al*. (2006) and Onovo and Ogaraku (2007). These authors worked on tigernut milk production and microorganism associated with their deterioration. Tigernut tubers from Angwan Kaje had higher fungal load than other locations while Keffi market had the least incidence. It is possible that the tigernut varieties sold at Keffi market are genetically resistant to fungal attack compared with the other markets (Eteshola and Orendu, 1996). It is also likely that marketers of these tubers at Keffi might be more careful with cleanliness and hygiene of their products. The percentage distribution of fungi deterioration of tigernut tubers from different locations showed that *Aspergillus flavus* had the highest frequency (29.69), while *Rhizopus oryzae* had the least frequency (14.06). As opined by Bhat (1988) the high frequency of occurrence of *Aspergillus flavus* might be due to production of a wide range of enzyme more than the rest of the other fungi. With such enzymes as cellulose, pectinase, ligninase, cutinase, suberinase etc the fungus could easily deteriorate plant cell wall causing rot.

The fact that the fluorescence bands produced correspond to aflatoxin B1 showed that the tubers infected with *Aspergillus flavus* and *A. niger* produced this secondary metabolite. Anton *et al*. (2004) reported the production of a mixture of mycotoxins by *Fusarium avennaceum*. Hence, compounds whose fluorescence colour intensities and retention factors (Rf) values differ from those of the available mycotoxin standards were observed in the sample extracts. As *R. oryzae* and *F. solani* infected sample extracts produce mycotoxins whose Rf value were 0.50 and 0.60, respectively. The presence of *Aspergillus flavus* and *A. niger* in our diets are of public health and economic importance. They produce mycotoxins like aflatoxin B1, which is hazardous to human and animal health (WHO, 1979). Factors which enhance the spread of fungi in Keffi L.G.A authority should be geared towards discouraging people from consuming infected tigernut as it could contain aflatoxin B1.

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Table 4: Developed Chromatogram of benzene: Acetonitrile extracts of *Aspergillus flavus, Rhizopus oryzae, Aspergillus niger and Fusarium solani*-infected tigernut tubers under ultra violet light.

	Standard Mycotoxins			***A. flavus***		***R. oryzae***		***A. niger***		***F. solani***	
	Aflatoxin B1	Aflatoxin B2	Ochratoxin	Control	Mean	Control	Mean	Control	Mean	Control	Mean
Rf Value	0.71	0.64	0.81	ND	0.71	ND	0.50	ND	0.71	ND	0.60
Colour and intensity under long wave length (365nm)	Blue and high	Blue and high	Green and low	ND	Blue	ND	ND	ND	Blue	ND	ND
Colour and intensity under short wave length (254nm)	Blue and high	Blue and high	Green and low	ND	Blue	ND	ND	ND	Blue	ND	ND
Colour after spraying with alcoholic aluminium chloride	NA	NA	Blue	ND	ND	ND	ND	ND	ND	ND	ND

Keyword: ND = Not detected, NA = Not applicable