JEB
Journal of Environmental Biology

Journal Website : www.jeb.co.in
E-mail : editor@jeb.co.in
ISSN: 0254-8704 (Print)
ISSN: 2394-0379 (Online)
CODEN: JEBIDP

Aspergillosis and Aflatoxicosis associated with tubers and leaves of sweet potatoes (*Ipomoea batatas*) in Osun state, South Western Nigeria

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Publication Info

Paper received:
29 April 2013

Revised received:
03 January 2014

Re-revised received:
19 August 2014

Accepted:
11 September 2014

Abstract

Tubers and leaves of sweet potatoes (*Ipomoea batatas*) are often contaminated with *Aspergillus* infection due to aflatoxin production. The present study was carried out to investigate the extent of in-vivo aflatoxin production due to *Aspergillus* infection, in different genotypes of sweet potatoes with different morphological and phenotypic characteristics. The genotypes selected were TIS 8441, CIP Tanzania, TIS 8164, TIS 2532.OP1 .13, TIS 87/ 0087 as well as Anamo Adayeba Pupa (local breed). Optimal aflatoxins were produced under aerobic conditions, at 30 ºC and high inoculum density during five days of incubation period. A strong relationship was observed between inoculum density and aflatoxin concentration. The physical and biochemical composition of the tubers and leaves of Anamo Adayeba Pupa makes it susceptible to *Aspergillus* infection and aflatoxin production. The fresh and dry leaves of Anamo Adayeba pupa, contained 2.208 ppb and 2.18 ppb of aflatoxins however, high levels of Aspergillosis was produced in the tubers with negligible amount of aflatoxins, however TIS 8441, CIP Tanzania, TIS 8164, `TIS 2532OP.1.13 were resistant to *Aspergillus* infection and aflatoxin production. The positive regression model showed correlation coefficient (R) value of 0.912 and 0.906 in fresh and dried leaves of Anamo Adayeba Pupa. A significant and positive correlation was found between *Aspergillus* infection and aflatoxin contamination .

Key words

Aflatoxicosis, Aspergillosis, *Aspergillus flavus*, *Ipomoea batatas*

Introduction

Ipomoea batatas commonly known as sweet potato belongs to family Convolvulaceae. It is an edible tuberous roots with high nutritional value. It is rich in carbohydrate and dietary fibers and provides a rich source of energy (O'hair, 2008 ,Scott and Maldona, 2009). In addition they have substantial amount of vitamins and minerals also. However, studies have reported presence of certain anti nutrients, like phytate, oxalate and tannins (Jonathan *et al.,* 2012). Tubers and leaves of sweet potato have also been found to be contaminated with microflora which are of air and soil origin (Witkoskha *et al*., 2011,Oyewale, 2012b, 2014). Aspergillosis as well as Aflatoxicosis have been found in major crops grown in United States (Chen *et al*., 2002), ground nuts in Nigeria, (Akano *et al*., 2002 and Garri, (Ogundero, 1982), milk and milk products (Oluwa Femi, 2012). Due to their harvest and storage methods, some of these microflora produce aflatoxins which have been classified as potent, carcinogens, mutagens and tetratogens secondary metabolites, produced primarily by two fungal species, *Aspergillus flavus* as well as *Aspergillus parasiticus* which posses a significant threat to crops, human as well as animal health (Bbosa *et al*., 2013; Bhatnagr *et al*., 2006; Cho *et al*., 2008; NAFDAC – IITA, 2013, Windham and William, 1998). The keeping quality of tubers and leaves is directly related to conditions at harvest and conditions before sale in the market, as well as length of storage at home (Oyewale 2012a). *Aspergillus flavus* is an important pathogen affecting various crops in Nigeria, and other parts of the world. It accounts for huge economic loss of major food crops grown in India and Nigeria (Priyanka *et al.,* 2012; 2014; IITA, 2013). Infection of maize kernel

by *Aspergillus flavus* and subsequent accumulation of aflatoxins is a chronic economic and health safety problems in south eastern United States (Brown *et al.,* 1993). The Nigerian Universities and Polytechnics in collaboration with International Institute of Tropical Agriculture, National Agency for Food and Drug Administration and Control, Raw Materials Research and Development Council of Nigeria, several other Government Agencies and Private Organizations are spreading awareness of potential risk and harzard of aflatoxin exposure.

Mycotoxins are low molecular weight toxic secondary metabolites produced by certain strains of filamentous fungi like *Aspergillus , Fusarium* and *Penicillium* species under favourable moisture and temperature conditions (Cardwell, 1995; Negedu, *et al.*, 2011; Azeez *et al.,* 2012; Vargai *et al.*,2009; 2011).They are usually produced during production, harvest, transportation, storage and food processing. In Nigeria, strong evidence of direct effect of mycotoxins on human health has been reported. In light of the above, the present study was carried out to investigate the extent of *Aspergillus* infection and aflatoxin contamination in sweet potato genotypes.

Materials and Methods

The present study was carried out in Boripe, Olorunda and Osogbo Local Government Area. Fields were cleared with tractors and ridges were constructed on the fields. Different varieties of sweet potatoes were then planted at each location. The resistant varieties selected for the trials included of TIS 8441, CIP Tanzania, TIS 8164, TIS 2532OP1.13, TIS 87/0087, red local variety while Anamo Adayeba Pupa local breed was also tried along with the resistant varieties. The crop was weeded occasionally and closely monitored for a period of 3 months.

Leaf samples of each sweet potato variety were carefully removed on weekly basis and kept in small polythene bags labelled and taken to the Pathology and Analytical Microbiology Laboratory for mycobiological analyses and quantification of aflatoxins in all the samples.

Tubers of sweet potato were carefully uprooted from the soil with cutlass and each breed line were carefully kept in small baskets and labelled. The pathogens were isolated from infected leaves and tubers. Infected tubers and leaves were surface sterilized, with 99% ethanol and rinsed five times with distilled water and incubated at room temperature for five days. Conidia which developed profusely from the surface of the tubers were removed with a sterile needle and inoculated on sterile potato dextrose agar medium.

In-vivo experiments were carried out to test the pathogenicity of fungus. Peeled sweet potato disc about 10x 10x50 mm were removed from mid portion of the tubers and surface sterilized and inoculated with hyphae or spore suspension of fungal isolate and incubated at room temperature in a petridish. After 4 weeks, each disc was sectioned with a freezing microtome and observed under digital photo-microscope for mycobiological study (Ogundana, 1977). The fungal isolates were mounted under microscope and the shapes and sizes of spores, hyphae as well as conidia were observed. The reactions of fungal isolates to sugar fermentation and different stains were noted using identification keys provided by (Allsop *et al*., 1981), and photographs were taken with a digital photo-microscope.

Sweet potato leaves were finely chopped and 10g of each sample was mixed with 50 ml of 70% methanol solution and centrifuged for 3 min. The mixture was allowed to settle and the filterate was used further for spectral analysis.

100µl of each sample and standard was added in appropriate well, containing 200µl of the conjugate. 100µl of the content from each dilution well was transferred into their corresponding antibody coated well and incubated for 15 min at room temperature. The contents of antibody wells were shaken vigorously, filled with distilled water and discarded out. The above procedure was repeated five times. 100µl of substrate was measured into each microwell strips using an eight channel pipette and incubated at room temperatures for 5 min. Finally 100µl of stop solution was pipette into micro well strips and checked for colour change. The bottom of micro wells were wiped with a dry towel, such that there was no fluids remained; the plate was taking into the micro-well reader using a 450 n.m filter. Air bubbles were eliminated, as they could affect the analytical results.

Dry samples of infected and healthy tubers were used for spectral analysis. Approximately, 0.2g of healthy and infected tuber samples were powdered in mortar and pestle and two drops of liquid paraffin (nujol , Mull) was added to make a creamy paste. The paste of each sample was determined for (a) tubers infected with *Aspergillus flavus* and (b) healthy and sound tubers. The spectra of various functional groups determined using the Buck Scientific model. I.R. spectrophotometer. (Oyewale 2012b).

Data of optical density and aflatoxin concentration was used to obtain regression equation and correlation co-efficient of fresh and dried leaves of sweet potatoes using SPSS computer software.

Results and Discussion

Poverty level among Nigerians with associated increase in prices of food commondities, ignorance about potent health risks of being exposed to pathogenic microorganisms and loss of nutritional health benefits inherent in tubers have made consumption of low quality tubers inevitable. In the present study, five prominent fungal species isolated and identified were *Mortierella ramanniana, Mucor pusillus, Erysiphe polygoni, Pennicillium* species and *Aspergillus flavus* (Table 1). Mycobiological analysis confirmed the growth of *Aspergillus*

flavus and presence of aflatoxins in leaves of Anamo Adayeba Pupa. *Aspergillus flavus* was distinguished due to the presence of primary and secondary sterigmata, secondary sterigmata bearing single conidiophores and sporangiophores (Fig1c).

The morphological and biochemical characteristic of different types of *Aspergillus flavus* isolated and identified from infected tubers, infected tubers, fresh leaves and dried leaves of Anamo Adayeba Pupa are presented in Table2. Seven types of *Aspergillus flavus* were identified in the present study utilized sucrose, glucose, fructose as well as maltose, however they did not utilize lactose .Environmental factors such as temperature and humidity had profound influence on the rate of *Aspergillus* infection and aflatoxin production. During *in-vivo* studies, optimum level of aflatoxin was produced under aerobic conditions at a temperature of 30 ºC and high inoculums density. The type of *Aspergillus flavus* that infected the fresh (fL_i, fL_{iii}) and dried leaves (DL_i, DL_{iii}) of Anamo Adayeba Pupa were physiologically different from that which infected the tubers (Tb_i, Tb_{ii} and Tb_{iii}) (Table 2). Hence this accounted for differences in their aflatoxin production potential. However, non aflatoxigenic *A. flavus* strain infected the tubers , while it was the aflatoxigenic *A. flavus* strain that infected the fresh and dried leaves of Anamo Adayeba Pupa. It was also noted that *A. flavus* that infected tubers did not produce aflatoxins as the tubers could develop anti aflatoxin inhibitors. The resistant breed lines used in this study, however contained genes which were resistant to fungal invasion and aflatoxin production. It can also be deduced from the present study that potential of *A. flavus* to produce aflatoxins is therefore dependent upon the gene clusters of the fungus, physiology of the organisms as well as prevailing environmental conditions. Analysis of samples revealed that *Aspergillus flavus, Pencillium* species, *Mortierella*

Table 1 : Fungi detected in leaves and tubers of Anamo Adeyeba Pupa

Leaves/Tubers	Fungal isolates				
	Aspergillus flavus	***Penicillium* spp**	***Mortierella ramanniana***	***Erysiphe polygoni***	***Mucor pusillus***
Fresh leaves	++	-	-	-	-
Dried leaves	++	-	-	-	-
Fresh tubers	++	+	+	+	+
Rotten tubers	++	+	+	+	+

Table 2 : Morphological and biochemical characteristics of *Aspergillus flavus* isolated and identified from infected leaves and spoilt tubers of Anamo Adayeba Pupa

Source of pathogens	Tubers	Fresh leaves	Dried leaves
Strain Numbers	Tb_i, Tb_{ii}, Tb_{iii}	fL_1 fL_{iii}	DL_i, DL_{iii}
Morphological	Club like	Club like	Club like
Characteristics	Sterigmata	Sterigmata	Sterigmata
Biochemical characteristics			
Reactions to stains			
Copper sulphate	+	++	+++
Iodine	+	+	++
Fermentation reactions			
Sucrose	+	+	+
Lactose	-	-	-
Glucose	+	++	++
Fructose	+	++	++
Maltose	+	++	++
Fungi detected	*Aspergillus flavus*	*Aspergillus flavus*	*Aspergillus flavus*

+ = Positive reaction; ++ / +++ = Highly positive reaction; - = Negative reaction

Table 3 : Regression equation and co-efficient of determination for aflatoxin production on Anamo Adayeba Pupa

Biotic factor	Regression equation	R^2	R	Aflatoxin concentration
Fresh leaves Anamo Adyeba pupa and *A. Flavus*	Y = -0.0423x+1.7441	0.8316	0.912	2.208
Dried leaves of Anamo Adyeba pupa and *A. flavus*	Y= - 0.05 x +2.0173	0.8216	0.906	2.18

Statistical analysis at $P \leq 0.05$

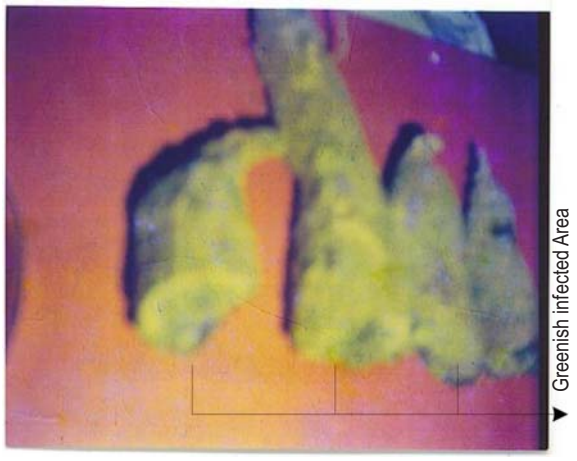

Fig. 1(a) : T.S. of sweet potatoes (*Ipomoea batatas*) infected by *Aspergillus flavus*

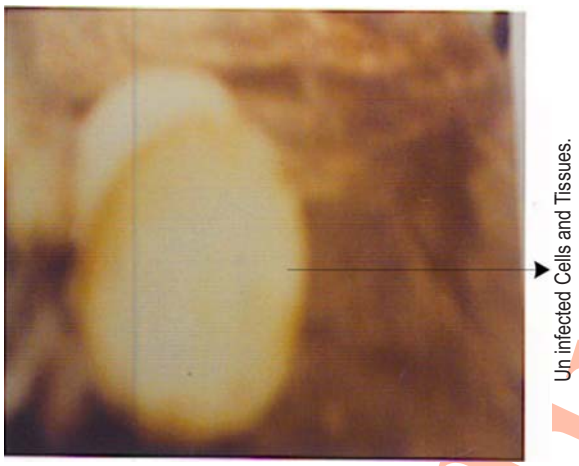

Fig. 1(b) : T.S. of healthy and sound sweet potatoes (*Ipomoea batatas*)

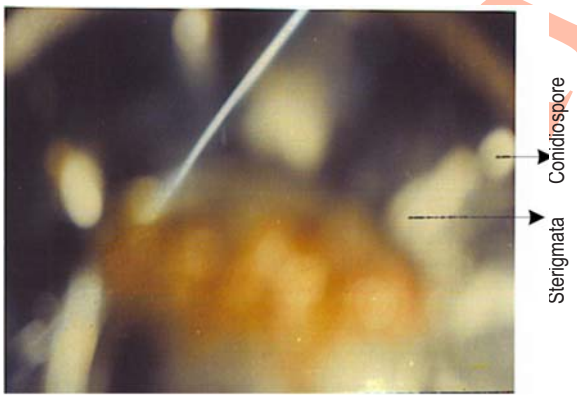

Fig. 1(c) : Micrograph of *Aspergillus flavus* isolated and identified from infected sweet potatoes.

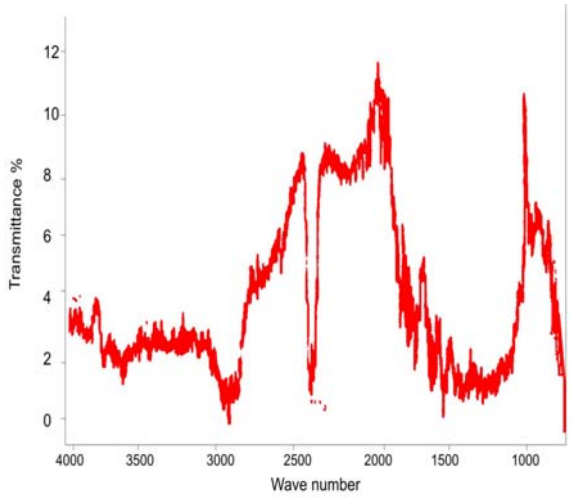

Fig. 2 (a): Infrared spectra of sweet potato tubers infected by *Aspergillus flavus*

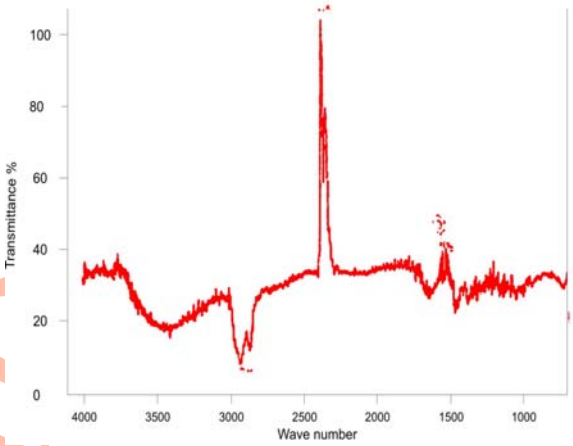

Fig. 2 (b) : Infrared spectra of healthy non-infected sweet potato tubers

ramanniana, Erysiphe polygoni as well as *Mucor pussillus* were of soil and air origin (Oyewale, 2012a; 2014).As a result of wind action, , soil particles and dusts are blown into the atmosphere , hence spores of soil borne fungus like *Aspergillus flavus* come in contact of with leaves. Chlorotic patches formed on the leaves of Anamo Adayeba Pupa, showed symptoms of *Aspergillus* infections and aflatoxin production due to development of germ tubes from conidia, followed by multiplication of fungal cells.Regression analysis revealed that during infection of fresh and dried leaves of Anamo Adayeba Pupa by *Aspergillus flavus* , a correlation co-efficient of 0.912 and 0.906 was obtained at $P \leq 0.05$ (Table 3). *In-vivo* study showed a significant positive correlation between *Aspergillus* infection and aflatoxin production during five days of incubation at 30 ºC. High contamination of Aspergillosis in leaves of Anamo Adayeba Pupa, lead to a corresponding increase in the level of aflatoxins. This could constitute potential hazards to man and animals feeding on the leaves. The levels of aflatoxins detected in fresh and dried leaves

of Anamo Adayeba Pupa collected from Kelebe farms were 2.208ppb and 2.18 ppb respectively (Table 3). The extracted sweet potato samples produced blue spots during laboratory analysis signaling low concentration of aflatoxins.

Akano *et al.* (2002) reported high level of aflatoxins ranging between 38.55 µg kg^{-1} to 205.60µg kg^{-1} in fourteen varieties of groundnuts in Northern Nigeria, which was less than those recorded in other parts of the world (Pakistan 24-800µg kg^{-1}, Mozambique 3-5500µg kg^{-1} and Brazil 5-22500µg kg^{-1}).

Oluwafemi (2012) documented aflatoxin in lactating mothers in two cities of south western Nigeria, however aflatoxin M_1 ranging between 2-187 µg ml^{-1} was detected in 17 samples of human breast milk (14.1%). In an another investigation, Akinyemi *et al.* (2011) reported aflatoxins ranging between 0.030-1.150 ppb in smoked fish sold in Abeokuta markets. While Jonathan *et al*. (2012) reported high concentration (0.0023 µg kg^{-1}) of aflatoxin B_1 in 9 month stored samples of sweet potato chips. The type of aflatoxins detected in fresh and dried leaves of sweet potato, sweet potato chip as well as smoked fish belonged to B1 class, however *Aspergillus flavus* was confirmed as source of aflatoxins.The concentration of aflatoxins detected in fresh and dried leaves of sweet potatoes, smoked fish as well as sweet potato chips were significantly less than that which could cause health harzards to man and live stocks animals feeding on them. However, bioaccumulation of aflatoxins with increasing incubation period could be harmful and dangerous to man and animals. The permissible limits of aflatoxins in food products is between 4.0-30 ppb depending upon country (FDA, 2009a; FDA, 2009b ; Williams *et al.,* 2004).

The morphological and biochemical characteristics of individual breed lines have profound influence on their potentials to be susceptible to *A. flavus*. In the present investigation, Anamo Adayebe Pupa was susceptible to *Aspergillus* infections and aflatoxin contamination, however TIS 8441, CIP Tanzania, TIS 8164, TIS 2532OP1.13 and TIS 87/0087 were resistant to *Aspergillus* infection and aflatoxin contamination.

Consumption of sweet potato infected by pathogens can cause liver cancer and hepatitis. *Aspergillus flavus* thrives well in the leaves of Anamo Adayeba Pupa. The presence of molds in leaves as well as tubers of sweet potatoes is undesirable. Fungal invasion and contamination often begins before harvest.

Recently, Jonathan *et al.* (2012) reported bio-deteriorating fungi and mycotoxins in fresh and stored chips of sweet potatoes. The study showed that storage of sweet potato chips increased moisture content, while carbohydrate, protein and fat content significantly decreased in stored samples.

Further, mycoflora examination revealed the presence of *A. tamari,A. niger, A. flavus, P.chrysogenum, F. compacticum and Saccharomyces* species. Presence of these mycoflora stimulated the production of mycotoxins, especially aflatoxin B_1 In stored *Ipomoea batatas* along with aflatoxin B_2, G_1 and G_2. Genotypes of sweet potatoes, drought, soil types and insect activities are important factors determining the likelihood of pre-harvest and post-harvest contamination. Enviromental factors such as, temperature ranging between 24°C-35°C and high relative humidity enhances *Aspergillus* infection and aflatoxin contamination. Spectrophotometric analysis of healthy sweet potatoes showed maximum four peaks at 1400 cm^{-1}, 2300 cm^{-1}, 2800 cm^{-1} and 2900 cm^{-1}, while infected sweet potato tubers showed several peaks at 900 cm^{-1}, 1100 cm^{-1}, 1300 cm^{-1}, 1400 cm^{-1}, 2400 cm^{-1}, 2700 cm^{-1}, 2900 cm^{-1}, 3400 cm^{-1}, 3800 cm^{-1} and 3900 cm^{-1} respectively. Further analysis revealed that healthy sweet potato tubers contained -CH- group, while infected tubers contained several intermediates products like -OH-, -CH- as well as glycosidic bonds, which were signs of enzymatic biodegradation of tubers (Fig. 2a). The result obtained in the present study is in agreement with the previous study of Kizil *et al.* (2002) ,which states that Infra red spectra revealed the structural mordification that could affect the functional and nutritional properties of food.

Multiple linear regression equation showed positive correlation between *Aspergillus* infection and foliar damage, to the extent of 83% (R^2 = 0.8316 and 82% , R^2 = 0.8216) hence, reducing the nutritional food value in fresh and dried leaves of sweet potatoes (Table 3).

Farmers in several advanced and developing countries of the world often invest huge amount of money in production by using sophisticated drying and storage facilities. Despite huge investment in storage facilities, substantial loss occurs. Farming methods as well as poor storage facilities in most farms and markets makes sweet potatoes, like other root crops, prone to *Aspergillus* infection and aflatoxin contamination. Poor sanitary condition of farms , as they are located in the rural areas, enhances tuber rot and leaf infection by *Aspergillus flavus* and subsequent aflatoxin production in leaves of sweet potatoes. The problem of poverty, food insufficiency and insecurity, intertwined with aflatoxin contamination, calls for new effective strategies for tackling these immeasurable problems.

Acknowledgments

Author is grateful to the Director General and management of National Root Crop Research institute, Umudike for providing sweet potato varieties used in this research work. Special thanks and appreciation goes to the Government of Osun state for procuring some of the equipments used in this research work.

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