



Temperature, Relative Humidity and Incubation Period as Critical Factors in *Aspergillus Flavus* Growth and Aflatoxin B1 Production on Maize Grains

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Short Communication

Abstract

Maize, an important staple in Nigeria and a major feed ingredient in the poultry industry is prone to aflatoxin B1 contamination as a result of favourable environmental and storage conditions that promote the growth of *Aspergillus flavus* from which aflatoxin B1 evolves. This study was conducted to assess the effect of storage conditions (temperature and relative humidity) and incubation period (14 and 21 days) on the growth of *Aspergillus flavus* and subsequent aflatoxin B1 production on maize. In a completely randomized design, 40 kilograms of pure maize were divided into 4 treatments (T1 to T4) of 10 kg each while each treatment was again replicated twice. Treatment 1 was the control which is the maize that was not inoculated with fungi and kept in a well-ventilated room (average temperature and humidity of 26°C and 60% respectively). Treatments 2, 3 and 4 respectively contained maize that were inoculated with a pure strain of *Aspergillus flavus* but kept under the shed (average temperature and humidity of 33°C and 58% respectively), refrigerator (average temperature and humidity of 14°C and 65% respectively), and in the open air (average temperature and humidity of 32°C and 75% respectively). The maize was left to incubate for 14 and 21 days. The result of Aflatoxin B1 levels from T1 to T4 after 14 days showed significant ($p < 0/05$) difference in mean values for samples that were kept for 14 days (0.00, 20,856.50, 19,342.50 and 28,191.00 ppb) with the highest value recorded in Treatment 4 that were incubated in the open air. In sharp contrast to this, maize that was incubated for 21 days recorded significant differences in their mean values (0.00, 19,487, 481 and 12,253 ppb) from T1 to T4 but those incubated in the open air recorded lower aflatoxin B1 values (12,253 ppb) compared to the one under the shed (19,487.00 ppb). No aflatoxin was recorded in the control treatment. In conclusion, a temperature as low as 14°C will favour mould growth and aflatoxin production within a period not longer than 2 weeks. Also, more aflatoxin is produced in maize that is left in the open air or in the field where temperature and relative humidity are higher than under the shed or refrigerator which provides a cooler environment.

Keywords: Aflatoxin B1, Maize grains, Temperature, Relative Humidity, Incubation period, *Aspergillus flavus*

Introduction

Aspergillus flavus and *Aspergillus parasiticus* are the two main fungi species responsible for the production of aflatoxin (Dutta and Das, 2001). They are a major concern in the poultry industry and indeed a threat to food security worldwide. Contamination of maize by mycotoxin is of importance due to the competition of man and animal for the cereal. It is still the major energy source in poultry feed in Nigeria today (FAO, 2003). Maize is prone

to infection by *Aspergillus* species while on the field and in storage because the weather in the tropics favours the growth of the moulds.

Aflatoxins are a major concern in the poultry industry because of serious economic losses resulting from their consumption by animals and humans. Foodstuffs and feeds are mainly contaminated by *Aspergillus flavus*, resulting in the production of aflatoxin B1.

Stored food is mainly contaminated by fungi due to its ability to remain in the food chain as a pathogen and saprophytes before harvest and after harvest (Lahouar *et al.*, 2015).

Several methods have been developed to alleviate the toxigenic effect of aflatoxin B1 when ingested including the use of toxin binder, but a more practical approach may be the prevention of the production of aflatoxin on the food materials by the fungi through a careful study of factors that favour its production. Temperature, relative humidity and incubation time are critical factors that influence the growth of *Aspergillus* fungi (FAO, 2007). Fungi have been known to thrive well within the temperature range of 10-35°C while some species can develop with temperatures below or above this range (Dix and Wester 1995). The water content of the air which is termed relative humidity is another important factor in fungal growth and it has been shown that high relative humidity up to 60% and above favours the growth of *Aspergillus* and aflatoxin production.

While there have been a series of studies targeted at eliminating aflatoxin contamination in the food chain, information about conditions that favour the sprouting of *Aspergillus flavus* and aflatoxin production is still scanty. This research was conducted to determine the combination of factors that may encourage *Aspergillus flavus* growth on maize to determine how such factors could be altered to prevent contamination that results from the activities of the fungi in the field.

Materials and Methods

Study site

This research was conducted at the Biological Sciences Laboratory of the Tai Solarin University of Education, Ijebu Ode, Ogun State of Nigeria and the Plant Pathology Laboratory of the International Institute of Tropical Agriculture, Moniya, Ibadan, Oyo State of Nigeria.

Source of *Aspergillus flavus*

A pure toxigenic strain of *Aspergillus flavus* (N3228) was isolated in the plant pathology laboratory of the International Institute of Tropical Agriculture (IITA), Ibadan Nigeria. The pure strain of the fungi was preserved in 1ml of distilled water.

Experimental Design and Statistical Analysis

In a completely randomized design, 40kg of clean maize was divided into four (4) groups of 10kg maize per group and each group was replicated twice.

Group 1 is the control group which contained ten (10) kilograms of maize that is free of contamination and kept at normal room temperature of 25°C - 26°C and relative humidity of 65%

Group 2 contained ten (10) kilograms of inoculated maize kept under a tree with an average temperature and relative humidity range of 30.58°C and 72%, respectively.

Group 3 contained ten (10) kilograms of inoculated maize kept in the refrigerator with an average temperature and relative humidity of 14.36°C and of 69% respectively.

Group 4 contained ten (10) kilograms of inoculated maize kept under direct sunlight with average temperature and relative humidity of 33.19°C and 58% respectively.

The temperatures and relative humidity mentioned above were the average of what obtained throughout the period of incubation.

Preparation of maize inoculum

The maize containing the aflatoxin (inoculum) was prepared by the method described by Shotwell *et al.* (1966). A pure culture of *Aspergillus flavus* was obtained from IITA in 1 ml of distilled water. Sub-culturing of the fungi was done at the Department of Biological Sciences, Tai Solarin University of Education using Potato Dextrose Agar (PDA) as culturing

medium. The 1ml solution containing *Aspergillus flavus* was subcultured on 10 potato dextrose agar nutrient plates using the micropipette and allowed to grow in an incubator for 24 hours. Other reagents that were used in the laboratory include tween 20, ethanol and jik bleach.

Pure dry maize was purchased from Oke Aje market in Ijebu Ode, Ogun State of Nigeria. The maize grains were soaked for six hours in a big plastic bowl. After soaking, the grains were autoclaved at 121°C for 20 minutes. The purpose of autoclaving was to ensure that the maize was free from any microbes before being used for the intended purpose. 1ml of tween 20 was added to 1000ml of distilled water and thoroughly shaken for proper mixing.

The tween 20 mixture is then poured gently into each plate containing the fungi while the substrate is stirred gently with a spreader and poured back into the 1000ml jar and mixed vigorously. 10 plates of the fungi were poured into a 1000 ml jar. Spore count was done using a micropipette to charge the haemocytometer with spore solution and covered with coverslip, and counted under a compound microscope with objective lens X 40 magnification. Spore load obtained per ml was obtained by multiplying the average count by a factor of 50,000.

Aflatoxin in the maize grains was produced by inoculating the autoclaved maize with the fungus strain, *Aspergillus flavus* isolate (N3228 strain). 500ml of spore solution was poured into each 5kg bag of maize and shaken vigorously until no visible water was seen in the nylon. Samples of maize were then kept under different conditions representing the treatments for two (2) and three (3) weeks to allow for high production of aflatoxin in the maize samples. After two weeks' samples of maize showed high contamination characterized by dark greenish to black colouration. The mouldy maize grains were washed with a bleaching

reagent (Jik) to kill the remnant spores before drying the maize grains.

Harvesting of samples

Maize samples were harvested on 14th and 21st day after inoculation. The samples also showed varying degrees of contamination depending on the incubation condition. The maize grains from different incubation conditions were collected and washed under adequate preventive measures, using bleach reagent (Jik) to kill the active fungi spores on the maize. The maize was then sun-dried and taken to the laboratory for aflatoxin quantification.

Aflatoxin quantification

Portions of the dried grains were collected homogenized and ground into powder with sizes less than 2mm with test portion sizes of 200 g. A 20 g sub-sample from a bulk sample of 200g was ground and extracted with 100 ml of 70% methanol using a high-speed blender (Waring Commercial, Springfield, MO) for 3 minutes. The mixture was then passed through Whatman paper No. 1, the extract was collected in a 250 ml separating funnel and 100ml of distilled water was added to ease the separation. The solution was extracted twice with 25ml methylene chloride. Following separation, the methylene chloride layer was filtered through 40g of anhydrous sodium sulphate to remove residual water. The extract was collected in a polypropylene cup and evaporated to dryness in a fume hood. The residue was dissolved in 200ul of methylene chloride and either diluted or concentrated to allow accurate densitometry. Extracts and Aflatoxin standards were separated on thin-layer chromatography (TLC) plates (silica gel 60, 250 um) by development with diethyl ether-methanol-water (96:3:1), visualized under ultraviolet light, and scored visually for the presence or absence of Aflatoxin with a 2mg limit of detection. Aflatoxins were quantified using a scanning densitometer, CAMAG TLC Scanner 3 with win-CATS 1.4.2 software (Camag AG,

Muttenz, Switzerland) as described previously (Suhagiaet *al.*, 2006)

Data collection

Records of daily temperature and relative humidity were taken throughout the experimental period and were divided by the number of days to get the average at the end of the study. The temperature and relative humidity were measured with a digital hydro-thermometer which was placed at the site of each sample. The temperature was measured in degree Celsius ($^{\circ}\text{C}$) while the relative humidity was measured in percentage (%). The records of the relative humidity were taken to determine the amount of water vapor contained in air at a particular temperature

while the records of the temperature were taken to determine the hotness and coldness of the environment. The quantification/estimation of aflatoxin was conducted as described earlier using thin layer chromatography (TLC) with scanning densitometer (Camag TLC Scanner 4, ISO 9001/2015 certified, and Reg. No. SQS CH11688, Switzerland) at the International Institute of Tropical Agriculture (IITA), Ibadan.

Data analysis

Data obtained from aflatoxin quantification were analysed using the statistical analysis software (SAS) 2010 package while the means were separated using Duncan multiple range test.

Results and Discussion

Table 1: Effects of Storage Conditions on Aflatoxin Production on Maize

Storage condition	Temperature ($^{\circ}\text{C}$)	Relative Humidity (%)	Incubation time (day)	Aflatoxin B1 concentration (ppb)	Standard error of mean
T1 (control)	26	60	14	0.00 ^c	364.3
	25.5	65	21	0.00 ^c	1,266.10
T2 (under tree)	31	72	14	20,856.50 ^b	364.3
	31	71	21	19,487.00 ^a	1,266.10
T3 (refrigerator)	14	69	14	19,342.50 ^b	364.3
	17	71	21	481.00 ^c	1,266.10
T4 (open air)	33	58	14	28,191.00 ^a	364.3
	33	56	21	12,253.00 ^b	1,266.10

^{abc}: means on the same column with different superscript are significantly ($p < 0.05$) different

In this study, Aflatoxin B1 is the only metabolite considered for analysis because other aflatoxin such as B2, G1 and G2 were either not detected or only left traces during quantification. The result of the analysis of the concentration of aflatoxin in the maize samples kept under different conditions showed significant ($p < 0.05$) differences as seen in Table 1.

From Table 1, no aflatoxin was detected in the maize samples that were not inoculated with *Aspergillus* but kept at normal room temperature of 26°C and relative humidity of 60 and 65% during 14 and 21 days

respectively. However, the highest aflatoxin B1 recorded (28,191 ppb) was in treatment 4 which was kept in the open air with a temperature of 33°C and relative humidity of 58% and an incubation period of 14 days.

Maize from treatment 2 and 3 had similar values of aflatoxin concentration even though temperatures and relative humidity values were not exactly the same, their incubation period (14 days) was the same. For maize samples that were incubated for 21 days, there was no significant ($p > 0.05$)

difference in the aflatoxin concentration across the treatments. The values of aflatoxin in maize kept at room temperature as control and the one in the refrigerator for the same incubation period of 21 days were not significantly ($p>0.05$) different.

From the foregoing, an incubation time of 14 days and a temperature as high as 33°C appears to be most favourable in the production of aflatoxin by *Aspergillus flavus*. Different authors have reported different findings in similar studies. Schindler *et al.* (1967) reported that maximal production of aflatoxin on wort media occurred at 24°C whereas the optimal temperature for aflatoxin B1 production was 35°C. Amani *et al.* (2016) reported that the maximum accumulation of aflatoxin B1 (2.26 ppb) was observed at 37°C and 99% relative humidity after 7 days of incubation. Diener and Davis (1967) concluded that the limiting low temperature for visible growth and aflatoxin production by *Aspergillus flavus* was 13±1°C for 21 days at 97 – 99% relative humidity while the maximum temperature for aflatoxin production was 41.5±1.50C for 21 days at 97 to 99% relative humidity

From the findings here, even at temperatures as low as 14°C and 69%

relative humidity, aflatoxin production was still as high as 19,342.50 ppb. At 21 days, however, aflatoxin production in maize samples incubated in the refrigerator at a temperature of 17°C was not different from the result obtained from the control group statistically.

Conclusion and Recommendation

In conclusion, maximum aflatoxin B1 production was achieved within 14 days of incubation as values obtained for 14 days were generally higher than values obtained for 21 days. Also, maize samples that were incubated in the open air at 33°C had the highest concentration of aflatoxin followed by the one in the refrigerator, under the shed and the control in that order. It is therefore recommended that maize should be kept in storage below 10°C to avoid mould growth and aflatoxin production.

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