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# Potential of using host plant resistance, nitrogen and phosphorus fertilizers for reduction of *Aspergillus flavus* colonization and aflatoxin accumulation in maize in Tanzania

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ABSTRACT

Aflatoxin contamination (AC) in maize, caused by the fungal pathogen *Aspergillus flavus* (Link), starts at pre-harvest stage. Hence, interventions that reduce entry and development of *A. flavus* in the field are required. Trials were carried out at Seatondale and Igeri, to evaluate the effects of nitrogen and phosphorus fertilizer combinations, hereafter referred to as fertilizers, on *A. flavus* and AC in maize kernels. The main treatments were four combinations of N and P fertilizers (60 or 120 kg $Nha^{-1}$ with 15 or 30 kg $Pha^{-1}$) and sub-treatments were of six popular maize hybrids. Plants at 50% silking were inoculated with the fungus through the silk channels. Grains from inoculated and control ears were analysed for AC using Enzyme Linked Immunosorbent Assay, and pathogen content quantified by Quantitative Polymerase Chain reaction. Higher AC (mean 6.51 μg $kg^{-1}$) occurred at Seatondale than Igeri (mean 0.45 μg $kg^{-1}$), probably due to low temperatures (8–23 °C) at Igeri. Fertilizers didn't cause significant differences in neither pathogen colonization nor AC at both sites. However, mean *A. flavus* accumulation, as measured by pathogen host DNA ratio, was thrice (0.16) as high in sub-optimal fertilizer conditions compared to optimal fertilizer rate (0.05). All hybrids were susceptible to *A. flavus* and AC, though a difference in AC was noted among the hybrids at both sites. PAN 691 showed the highest AC (14.68 μg $kg^{-1}$), whereas UHS 5210 had the lowest AC (1.87 μg $kg^{-1}$). The susceptibility varied among the hybrids and was mostly associated with ear droopiness, husk tightness, days to 50% silking, 50% pollen shed, Anthesis to silking interval, diseased ears, insect damaged ears, kernel texture, dry matter, grain filling, ear height, kernel ash content and kernel moisture content. At Seatondale, *A. flavus* accumulation was positively correlated with aflatoxin ($r = 0.606$), and both *A. flavus* accumulation and AC were positively correlated with diseased ears. Selection and growing of less susceptible varieties under optimal fertilizer regime offer ideal strategy for sustainable reduction of *A. flavus* and aflatoxin contamination in maize at pre-harvest.



## 1. Introduction

Maize (*Zea mays* L.) is the most important cereal crop in Tanzania with an estimated annual per capita consumption of more than 112.5 kg per person, and a national wide annual consumption of more than three million tons (Bisanda et al., 1998). Unfortunately,

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the crop is highly vulnerable to contamination with mycotoxins including aflatoxins and fumonisins. Aflatoxins, which are produced by the fungal pathogen *Aspergillus flavus* (Link), continue to be of great concern to food safety and security, trade, and maize value chain, owing to their widespread contamination in maize and their adverse effects on animal and human health (Abbas et al., 2011; Miller, 2008). The production and accumulation of aflatoxin on naturally infected maize ears is therefore an important food safety concern, especially to people relying on maize as staple food.

A number of factors contribute to *in planta* aflatoxin production, including genotype, environment, inadequate pre-harvest and storage practices, as well as the presence of storage insect pests and the occurrence of other fungi (Mehan et al., 1986; Cotty and Jaime-Garcia, 2007; Hell et al., 2010; Warburton and Williams, 2014). Combination of some or all of these factors ultimately increases the risk of aflatoxin production in field crops, stored food, and feed (Cardwell and Henry, 2004). As such, aflatoxin contamination at pre-harvest is more severe when maize varieties with genotypic characteristics that predispose them to aflatoxin are grown under stress conditions and poor agronomic practices (Cardwell and Henry, 2004; Hell et al., 2008).

The use of good agricultural practices (GAP) has helped to manage *A. flavus* and aflatoxin contamination at pre-harvest in maize (Diener and Davis, 1987). Other management strategies include the use of resistant varieties, application of atoxigenic *A. flavus* fungal strains at time of sowing, and timely harvesting (Brown et al., 2003; Clements and White, 2004; Kaaya and Kyamuhangire, 2006; Atehnkeng et al., 2008;Yan-ni et al., 2008). However, despite the importance of maize in national and household food security and income generation, maize in Tanzania is mainly produced by smallholder farmers. The farming system is associated with poor production technologies, the use of disease susceptible varieties (Mushongi, 2010), and low use of inputs, such as low application of nitrogen (N) and phosphorus (P) fertilizers with application rates averaging 20 kg $Nha^{-1}$ and 10 kg $Pha^{-1}$ while the recommended rate varies from 60 to 120 kg $Nha^{-1}$ and 20–30 kg $Pha^{-1}$ (Lyimo, 2006).

Adequate supply of plant nutrition is one of the potential GAP against *A. flavus*. The N and P fertilizers are known to reduce the risks of fungal colonization in maize kernels and the production of aflatoxin (Lillohoj and Zuber, 1975). For instance, a number of studies have linked increased aflatoxin accumulation in grains with suboptimal levels of N fertilizer application (Lillohoj and Zuber, 1975; Payne et al., 1989). The application of sufficient N and P often speeds up the growth of maize seedlings and roots, making the plant healthier and stronger against damage from weak pathogens.

Host plant resistance offers the ideal foundation for integrated management of *A. flavus* in maize, despite the relatively low heritability of resistance to *A. flavus* and aflatoxin contamination (Brown et al., 2003; Clements and White, 2004; Warburton and Williams, 2014). Studies in Kenya showed that maize varieties that have good husk cover and ears that droop at physiological maturity had less aflatoxin contamination, as these attributes prevent moisture penetration while in the field before the crop is harvested (Aflacontrol Project, 2010).

It is envisaged that integrated suite of intervention measures remains to be the ideal strategy for sustainable reduction of *A. flavus* and aflatoxin contamination in maize at pre-harvest. This would entail development and implementation of more than one strategy in the context of integrated disease management (IDM). As such, the present study aimed at (i) assessing the modulating effects of nitrogen and phosphorus fertilizers on *A. flavus* and aflatoxins development in maize kernels, and (ii) evaluating the genetic susceptibility of selected maize hybrids to *A. flavus* and aflatoxin accumulation when grown in two contrasting environmental conditions.

## 2. Materials and methods

### 2.1. Location and establishment of field trials

Field trials were carried out from 18th December 2012 to 5th May 2013 and 16th December 2012 to 1st July 2013 at two substations of Agricultural Research Institute - Uyole, namely Seatondale and Igeri, respectively. The sites were selected based on the prevalence of *A. flavus* and the level of maize aflatoxin observed from farmers' maize at pre and post-harvest as determined by an earlier study (Gnonlonfin et al., unpublished data). Seatondale is situated in Iringa district at 7° S, 35° E, altitude of 1560 m and annual rainfall of 750 mm with temperatures ranging from 12 to 28° C, whereas Igeri is located at 9° S, 34° E, about 40 km south of Njombe town. The area has an elevation of 2200 m and receives an average seasonal rainfall of 2000 mm with cool temperatures throughout the year ranging from 8 to 23° C. The soils at both sites are highly weathered and leached, frequently acidic and of relatively low fertility (Ngasongwa, 2007; URT, 2008).

#### 2.1.1. Soil sampling and analysis

Soil samples from each site were collected and analysed before planting to gauge the amount of N and P fertilizer to be applied. Soil sampling was done using simple randomized procedure. Through this method, the field was demarcated into homogenous sampling areas and 40 samples per depth were collected from 0 to 15, 15–30 and 30–60 cm using soil auger. Two composite samples per depth from each site were taken to ARI-Uyole for analysis of total N, available P, cation exchange capacity (CEC), exchangeable bases and determination of organic carbon, soil textural classes and soil pH following standard procedure (Okalebo et al., 1993).

For analysis, samples were air-dried on shallow trays in a ventilated area or forced air oven at 40 °C and then passed through a mechanical crusher to break up any clay clods. The crushed soils were freed from gravel, roots and larger organic residues by sieving through a 2 mm sieve. Soil samples were further ground in a mortar in order to pass through a 60 mesh screen for analysis of total N, organic carbon and available P. The total N and available P were determined by using Kjeldahl and Olsen Methods, respectively (Okalebo et al., 1993).

#### 2.1.2. Experimental design

A split-plot design, with seven replications, was used to evaluate the effect of N and P fertilizers application rates on the development of *A. flavus* and aflatoxin accumulation in six maize hybrids. The main plot treatments were four levels of N and P fertilizers and the sub plot treatments were six maize hybrids (i.e. UH 6303, UH 615, UHS 5210, UHS 5350, SC 627 and PAN 691). The four levels of N and P combinations were $N_1P_1$, $N_1P_2$, $N_2P_1$ and $N_2P_2$:

1. $N_1$ = Recommended rate of N application which is 120 kg $Nha^{-1}$ adjusted after soil analysis of soil samples from the site
2. $N_2$ = 60 kg $Nha^{-1}$ which is half of the recommended rate of N
3. $P_1$ = Recommended rate of P application which is 30 kg $Pha^{-1}$ adjusted after soil analysis of soil samples from the site
4. $P_2$ = 15 kg $Pha^{-1}$ which is half of the recommended rate of P

Each replication contained 24 plots (i.e. six varieties in each of the 4 main plots). The plot size was 2 rows of 3 m length each, with a plant spacing of 75 × 30 cm giving 11 plants per row.

*2.1.3. Fertilizer application and crop management*

Fertilizer application was done using Triple Super Phosphate (TSP, 46% P) in form of $P_2O_5$ and Calcium Ammonium Nitrate (CAN, 27% N). The two sources of N and P were blended into four levels based on soil analysis results and actual farmers' fertilizer use practices. The blended TSP and CAN were applied in planting holes where one half of the N rates were used. The maize plants were later side dressed, with remaining half of N, 21 days after seedling emergence. Field management practices, such as weeding and control of insect pests were non-experimental variables. The use of herbicides was prohibited to avoid effects on the fungal population, which could have interfered with the objectives of the study.

*2.2. The A. flavus isolation, screening and storage*

Maize cobs were collected from farmers' stores in 11 different localities. Fungal isolation was then carried out at Biosafety Laboratory Level II at Agriculture Research Institute (ARI-Mikocheni) in Dar es Salaam, Tanzania, following protocols established by BecA-ILRI. Briefly, maize kernels onto Potato Dextrose Agar (PDA) Petri dishes were incubated at 27 °C for seven days. Seventy-six strains of *A. flavus* were isolated and characterized by their Morphology (S or L strain) and Toxigenicity (extent & type(s) of toxin produced). Two site specific strains of *A. flavus* that had high and similar levels of aflatoxin B1 production were selected for field inoculation. The strains were stored in screw capped bottles of PDA slants kept at 4 °C until when retrieved for routine sub-culturing or field studies.

*2.3. The A. flavus inocula production and field inoculation*

*2.3.1. Inocula production*

Site-specific inoculum (local isolates) of *A. flavus* strains was produced at ARI-Mikocheni, following a protocol that uses autoclaved maize kernels as substrate. Briefly, 30 g of maize kernels were soaked overnight in 250 mL conical flasks containing 15 mL of distilled water (water: maize kernels = 1:2). The imbibed kernels were covered with aluminium foil, then autoclaved at 120 °C for 30 min and allowed to cool at 28 °C in the Biosafety II cabinet. Thereafter, sliced discs of PDA medium containing pure *A. flavus* colonies were inoculated in each flask using a sterilized surgical blade. The flasks were gently shaken to ease distribution of conidia into the kernels and were incubated at 30 °C for 10 days. The flasks were shaken daily for 10 days to facilitate aeration of the fungi. The *A. flavus* conidia were harvested from maize kernels by washing them using a phosphate buffered saline of 20% Tween 20. The inoculum was adjusted to $1 \times 10^8$ conidia/mL, using haemocytometer and stored at 4 °C. The temperature was maintained throughout transportation and during inoculation process, which was done within 2 days of inoculum production.

*2.3.2. Field inoculation*

Just before plants had reached 50% silking, each ear was shoot bagged using pollination plastic bags in order to: a) reduce exposure to foreign microbes between silking and inoculation, b) create barrier against any sources of pollination which might block the silk channels, c) create moist environment which made the silks viable for relatively longer time periods and d) ensure uniform kernels formation on cobs [Gnonlonfin and Mushongi, *pers. Comm*., 2013]. Artificial inoculation was carried out when entries had attained 50% silking within the plots and across the seven replications. Twenty-four hours before inoculation, the shoot bags were removed to allow excess moisture in the silks to dry. Five plants in each plot were identified, labelled, tagged and ear-inoculated with 1 mL of $1 \times 10^8$ conidia/mL suspension of *A. flavus* using a 5 mL needleless disposable syringe. The suspension was gently squeezed at the tip of maize ears using the tip of the syringe as a snout. Five additional plants that were not selected for inoculation were tagged and remained as un-inoculated controls. Disposable inoculation containers and materials were sterilized by immersion in 10% commercial bleach (i.e. 3.85% Sodium hypochlorite) and incinerated.

*2.4. Data collection and measurement of secondary traits*

*2.4.1. Anthesis to silking interval (ASI)*

Recording of flowering dates was done soon after an individual genotype within N and P treatment and across the seven replications had attained 50% pollen shed and silking. The ASI was computed as: ASI = Days to 50% pollen shed − Days to 50% silking (Mushongi, 2010).

*2.4.2. Ear placement, ear drooping and husk coverage*

Five plants were randomly selected from the two rows. Plant height (PH) was measured from the soil surface to the flag leaf. Ear height (EH) was measured from the soil surface to the shoot node where the highest ear was situated. The ear placement (EP) was computed as:

$$\mathrm{EP} = \frac{EH}{PH}.$$

Ear droopiness was assessed across the hybrids using an arbitrary scale of 1–4 as follows: 1 = completely drooped (180° from top stem), 2 = partially drooped (135°), 3 = horizontally placed (90°) and 4 = upright positioned (<45°).

Husk covers were evaluated by using an arbitrary scale of 1–5 where; 1 = very tight coverage, 2 = tight coverage, 3 = loose coverage, 4 = tight covers with open tips and 5 = loose covers with open tips (Mushongi, 2010).

*2.4.3. Harvesting and shelling of ears*

Harvesting of maize hybrids was done at physiological maturity when the grains in non-inoculated plants attained average moisture contents of ≤15%. Inoculated ears were harvested separately from the non-inoculated ears and taken to the shelling shelter for processing. Shelling of inoculated ears was done following a protocol from BecA-ILRI. Site specific samples of the same genotype from the fertilizer rates were separately shelled. This was done to avoid cross contamination. All materials and sample residues were completely incinerated. After shelling, grains from each inoculated and control plots were processed, packed and shipped to the BecA-ILRI Hub where they were stored at 4 °C cold room before further laboratory analyses.

*2.4.4. Determination of kernel moisture, texture, density and ash contents*

Average kernel moisture content of grains in each plot was determined during harvesting using grain moisture metre (DICKEY-John ® Corporation mini GAC® moisture tester). The assessment of kernel texture of individual varieties was done during harvesting using a scale of 1–5 to assess individual ears per each plot, where:

1 = Flint, 2 = Semi-flint, 3 = Hard dent, 4 = Dent and 5 = Soft dent.

Additionally, 300 kernels from each plot were used in the laboratory for the measurement of kernel density following the 'Law of flotation' whereby the samples were oven-dried to constant weight in an oven (Heraeus HANAU Joh. Achelis J. Johne 2800 Bremen/ Germany) set at a temperature of 70 °C. Thereafter, samples were immersed into 1000 mL of a graduated measuring cylinder containing 200 mL of distilled water; the difference in volume (i.e. volume of displaced water) was determined and used to calculate

the density of each sample as follows:

$$\text{Volume of sample (displaced volume)} = \text{Final volume} - \text{Initial volume}.$$

$$\text{Kernel Density}(g/mL) = \frac{\text{Weight of a sample(at constant weight)}}{\text{Displaced volume of a sample}}$$

Ash content was determined by complete burning of the samples at 400 °C for 30 min using a Blast Furnace (CAABOLITE Parson Lane, Hope Valley S33 6RB, England) and the weight of ash from each sample was recorded. Kernel ash contents (fraction) were calculated using the following formula:

$$\text{Ash Content} = \frac{\text{Weight of Ash}(g)\text{of a sample}}{\text{Constant weight}(g)\text{of a sample}}$$

#### 2.4.5. Assessment of diseases and insect pests damage

During harvesting, diseased and insect damaged cobs were recorded and percent ear rot and percent insect damage were calculated as:

$$\%\ \text{Ear rot} = \left(\frac{\text{Number ears diseased}}{\text{Total ear harvested}}\right)*100 \text{ and}$$

$$\%\text{Insect damage} = \left(\frac{\text{Number of damaged ears}}{\text{Total ears harvested}}\right)*100$$

### 2.5. Detection of A. flavus and quantification of aflatoxin

#### 2.5.1. Sample preparation

The analysis of Aflatoxin content and *A. flavus* was carried out at BecA-ILRI hub focusing on the detection of *A. flavus* and quantification of aflatoxin $B_1$. A total of 168 inoculated maize samples and 168 control samples from each site were analysed. Each variety had 28 inoculated (i.e. 4 N and P rates X 7 replications) and 28 non-inoculated samples (control samples). The samples were ground to fine particles, equal to the size of instant coffee, using a Romer Mill (Romer Series II® Mill, Grinding/Subsampling mill U.S. Patent ≠4679737: Australia). During milling, cross contamination between samples was controlled by passing a small portion of the samples through the mill before the complete sample was milled, followed by vacuuming the mills at the end of milling of each sample using a vacuum cleaner (LG 2000W).

#### 2.5.2. Quantification of aflatoxin $B_1$ using ELISA

Standard Enzyme Linked Immunosorbent Assay (ELISA) extraction protocol, as described by the manufacture was used. Briefly, sample extraction was carried out as follows: Five grams of a ground sample was dissolved into 25 mL of 70% methanol at a ratio of 1:5 (w/v). The dissolved sample was shaken at 2500 rpm for four minutes using a controlled environmental incubator shaker (New Brunswick Scientific Co., Inc. Edson, N.J., USA.) and allowed to settle. Aflatoxin B1 was extracted by mixing prepared samples on horizontal shaker (at 220 rpm at 25 °C for 4 min). From this extract, aflatoxin B1 was quantified using ELISA kit (aflatoxin B1 ELISA Quantitative, Helica Biosystems, Inc, Catalog♯ 941BAFLO1B1) by logit-log standard curve. All sample extracts were diluted for a second ELISA if above 20 μg/kg, and then those with concentration above 100 μg/kg were further diluted and reanalyzed using VICAM® according to manufacturer instructions as following.

#### 2.5.3. Aflatoxin B1 quantification by VICAM

Further dilutions were conducted on the above mentioned category of extracts. Prior to quantification these extracts were cleaned up using a fluorometer. In brief, 2.0 ml of diluted extract (0.2 g sample equivalent) was passed through an Aflatest®-P affinity column at a rate of 1–2 drops/second and the column was then rinsed twice with 5 ml of distilled water at the same rate. The aflatoxin B1 material bound to the affinity column was eluted with 1.0 ml HPLC grade methanol at the rate of 1–2 drops/second and the eluate was collected in a glass tube. One ml of Aflatest® developer solution was added to the eluate from the Aflatest-P column, mixed by inversion several times, and concentrations of aflatoxin B1 (μg/kg) were detected after 60 s using a VICAM fluorometer (Series-4EX, Source Scientific LLC, USA) calibrated with a blank of methanol, according to the standard manufacturer's protocol. The detection limit of this method was 2.0 μg/kg.

### 2.6. Determination of A. flavus biomass by extraction of total genomic DNA

Inoculated ground maize samples with their corresponding negative controls from the two trial sites were tested for *A. flavus* biomass using a Real-time Polymerase Chain Reaction (qPCR) (7900HT Fast Real-Time PCR System). The total genomic DNA (i.e. from the pathogen and the host) was extracted from *A. flavus* inoculated and non-inoculated samples using a modified protocol of maize flour DNA extraction (CIMMYT, 2005). The quantity and quality of DNA was determined using Nanodrop Spectrophotometer (L1-GOR, 430 DNA Analyser. Canada). Additionally, the quality of the extracted DNA was determined by Gel electrophoresis using 0.8% agar rose gel at 50 V for 1 h in 0.5 × (Tris-borate-EDTA) TBE buffer. The DNA samples were normalized to 20 ng/μL using ultrapure sterile water before being taken to qPCR.

### 2.7. Relative quantification of A. flavus biomass in the grains

The host/pathogen DNA ratio from *A. flavus* inoculated and non-inoculated grains samples were quantified by the SYBR green based real time PCR reaction. Two sets of primers were used (Mideros et al., 2009). The *A. flavus* specific primers targeting 73bp fragment of the internal transcribed spacer gene (ITS) were used. The pathogen to host ratio (P: H) and the fungal biomass in the infected maize samples were then calculated (Williams et al., 2013).

### 2.8. Data analyses

The data collected from each trial were subjected to Analysis of variance using GenStat software version 14. The differences in responses among factors and variates were considered significant at $p \leq 0.05$. Interactions between main treatments and sub treatments were tested for each variable. Mean separation (multiple comparisons test) among N and P rates and hybrids was done using Student Newman Keuls test at 5% level of significance. Simple linear correlation analyses were performed to determine the correlation between the level of aflatoxin accumulated in each sample with selected maize phenotypic characteristics, assessed in the field and after harvest. The characteristics included husk cover tightness, husk density, ear droopiness, open tips, kernel texture, kernel density, kernel moisture content, kernel ash, dry matter, plant height, ear height, days to 50% silking, days to 50% pollen shed, ASI, days to grain filling, days to physiological maturity and grain yield. Data of some of the variables used in correlation analyses were subjected to the Log $(x+1)$ or the Square root $(X+0.5)^{1/2}$ transformation prior to analyses. All data for correlation analyses were

averaged across the seven replications and Pearson product-moment correlation coefficient determined.

## 3. Results

### 3.1. Soils and weather conditions

Results from analysis of soil samples are presented in Table 1. Interpretations of plant nutrient requirement were done following the procedure described by Okalebo et al. (1993). Differences in weather conditions were observed during the conduct of the experiments at the two sites as shown in Figs. 1 and 2. Igeri had the lowest minimum and maximum temperatures (8–23 °C) compared to Seatondale (12–28 °C) during the entire season and a higher total rainfall (3300 mm) as compared to Seatondale (730 mm).

### 3.2. The A. flavus and aflatoxin contents in maize samples

The results of aflatoxin contents and *A. flavus* biomass are presented in Table 2. Variation of fungal biomass and aflatoxin levels between sites and genotypes was observed. The mean levels of aflatoxin accumulation were significantly lower at Igeri (0.45 μg $kg^{-1}$) compared to Seatondale (6.51 μg $kg^{-1}$). The levels of aflatoxin in samples collected from Seatondale were not significantly different across the N and P fertilizer combinations, although the trend showed a reduction in aflatoxin concentration when the optimal rate $N_1P_1$ was used. For instance, at Seatondale grains fertilized with $N_1P_1$ accumulated 35.65% less aflatoxin (4.77 μg $kg^{-1}$) than when sub-optimal $N_2P_2$ was used (8.61 μg $kg^{-1}$). In addition, fertilizer rate $N_1P_2$ showed more variation in the aflatoxin levels compared to the rest of the fertilizer treatments.

Significant differences in aflatoxin concentrations were found among the hybrids grown at Seatondale. Hybrid PAN 691 significantly accumulated the highest mean aflatoxin concentration of 14.68 μg $kg^{-1}$, whereas hybrid UHS 5210 accumulated the lowest mean concentration of 1.87 μg $kg^{-1}$. At Igeri there were no significant differences in aflatoxin concentration among the hybrids, though hybrid UHS 5210 again accumulated the lowest mean aflatoxin concentration of 0.22 μg $kg^{-1}$ and hybrid SC 627 had the highest mean aflatoxin concentration of 0.84 μg $kg^{-1}$.

The N and P rates did not cause significant difference in *A. flavus* colonization in both sites though mean accumulation of *A. flavus* was found to be thrice as much in $N_2P_2$ compared to $N_1P_1$. The six hybrids did not show significant differences in terms of *A. flavus* accumulation in both sites, although hybrid PAN 691 had the highest mean pathogen host DNA ratio of 0.22 at Seatondale while hybrid UH 6303 had the highest mean pathogen host DNA ratio of 0.02 at Igeri.

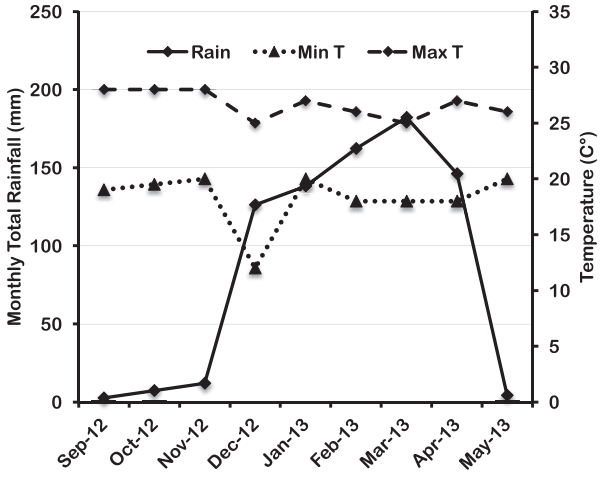


**Fig. 1.** Monthly rainfall, minimum and maximum temperatures for the period September 2012 to May 2013 at Seatondale.
Source: Meteorological station at Iringa, Tanzania.

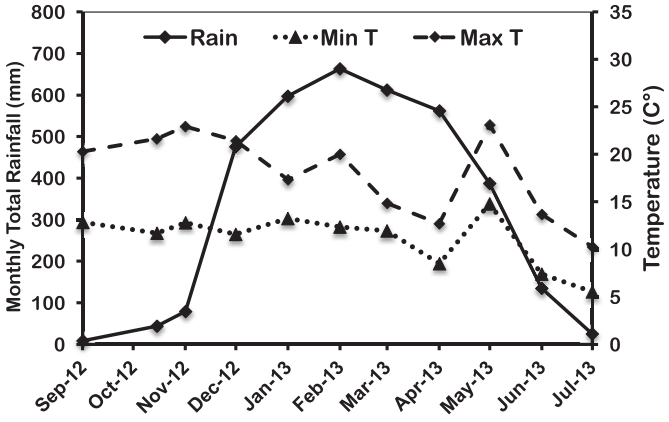


**Fig. 2.** Monthly rainfall, minimum and maximum temperatures for the period September 2012 to May 2013 at Igeri.
Source: Meteorological station at Njombe, Tanzania.

### 3.3. Correlations between A. flavus, aflatoxin and other variables

#### 3.3.1. Correlation between A. flavus, aflatoxin and field variables

Correlation coefficients among *A. flavus* in the grains, aflatoxin content in the grains, and field variables at Seatondale and Igeri are presented in Table 3. The *A. flavus* accumulation in the grains was positively correlated with aflatoxin (r = 0.606) at Seatondale. At

**Table 1**
Mean soil analysis results before establishment of trials for Seatondale and Igeri.

| Site[a] | Soil pH[b] | | PSD[c] (%) | | | TN[d] (%) | OC[e] (%) | P[f] | CEC[g] | Exchangeable bases (cmol + $Kg^{-1}$) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $H_2O$ | KCl | Clay | Silt | Sand | | | | | Ca | Mg | K | Na |
| 1 | 5.79 | 5.40 | 18.00 | 26.25 | 55.08 | 0.11 | 1.10 | 16.49 | 16.39 | 0.80 | 0.81 | 0.75 | 0.73 |
| 2 | 5.44 | 4.87 | 16.61 | 27.56 | 55.83 | 0.28 | 3.58 | 7.05 | 14.73 | 0.61 | 0.65 | 0.93 | 0.63 |

[a] Site: 1 = Seatondale and 2 = Igeri.
[b] pH = soil reaction is an indication of the acidity or alkalinity of soil and is measured in pH units (Scale 1–14).
[c] PSD = Particle size distribution.
[d] TN = Total Nitrogen.
[e] OC = Organic Carbon.
[f] P =Phosphorus content in mg/Kg.
[g] CEC = Cation Exchange Capacity in Centi −mol + per kg.

**Table 2**
Mean *A. flavus* and Aflatoxin for fertilizers and Hybrids treatments at Seatondale and Igeri.

| Treatments | Seatondale | | | Igeri | | |
|---|---|---|---|---|---|---|
| | *A.flavus* ([d]P:H ratio) | Aflatoxin (μg kg$^{-1}$) | Aflatoxin (μg kg$^{-1}$)[a] | *A.flavus* (P:H ratio) | Aflatoxin (μg kg$^{-1}$) | Aflatoxin (μg kg$^{-1}$)[a] |
| Fertilizers | | | | | | |
| 1. $N_1P_1$ | 0.05a[b] | 4.77a | 0.45a | 0.00a | 0.25a | 0.08a |
| 2. $N_1P_2$ | 0.04a | 6.86a | 0.54a | 0.01a | 0.34a | 0.11a |
| 3. $N_2P_1$ | 0.03a | 5.81a | 0.53a | 0.01a | 0.34a | 0.11a |
| 4. $N_2P_2$ | 0.16a | 8.61a | 0.61a | 0.02a | 0.88a | 0.18b |
| SE | 0.07 | 1.50 | 0.06 | 0.01 | 0.17 | 0.18 |
| LSD | 0.02 | 4.45 | 0.17 | 0.03 | 0.49 | 0.05 |
| Hybrids | | | | | | |
| 1. UH 615 | 0.01a | 7.49a | 0.52ab | 0.01a | 0.34a | 0.11a |
| 2. UH 6303 | 0.06a | 7.03a | 0.67bc | 0.02a | 0.42a | 0.14a |
| 3. UHS 5210 | 0.01a | 1.87a | 0.25a | 0.01a | 0.22a | 0.08a |
| 4. UHS 5350 | 0.03a | 4.10a | 0.34ab | 0.00a | 0.42a | 0.12a |
| 5. SC 627 | 0.10a | 3.89a | 0.51ab | 0.00a | 0.84a | 0.14a |
| 6. PAN 691 | 0.22a | 14.68b | 0.91c | 0.01a | 0.46a | 0.14a |
| SE | 0.09 | 2.03 | 0.09 | 0.01 | 0.24 | 0.03 |
| LSD | 0.24 | 5.69 | 0.25 | 0.03 | 0.68 | 0.08 |
| Mean | 0.07 | 6.51 | 0.53 | 0.01 | 0.45 | 0.12 |
| Interaction[c] | NS | NS | NS | NS | NS | NS |

[a] Aflatoxin = Means were transformed before correlation analyses.
[b] Means within columns and within treatments followed by common letters are not significantly different at $p \leq 0.05$, according to Student Neuman Keul's test. Values are means of 7 replications.
[c] Interaction: NS= Not significant at $p \leq 0.05$, P:H= Pathogen: Host ratio.

**Table 3**
Correlation coefficients between *A. flavus*, Aflatoxin and field variables for Seatondale and Igeri sites.

| Field variables | Site | | | |
|---|---|---|---|---|
| | Seatondale | | Igeri | |
| | *A. flavus* | Aflatoxin (μg kg$^{-1}$) | *A. flavus* | Aflatoxin (μg kg$^{-1}$) |
| *A. flavus* (P:H DNA ratio) | – | | – | |
| Aflatoxin (μgkg$^{-1}$) | 0.606** | – | 0.051 | – |
| Diseased ears (%) | 0.622** | 0.503** | −0.239 | 0.488* |
| Insect damage (%) | 0.146 | 0.465* | −0.16 | −0.211 |
| Ear droop (score) | −0.282 | −0.477* | −0.209 | −0.199 |
| Husk tightness (score) | 0.228 | 0.424* | −0.214 | −0.088 |
| Open tips (%) | 0.091 | 0.332 | 0.042 | 0.093 |
| Lodged plants (%) | −0.218 | −0.253 | 0.084 | 0.115 |
| Anthesis to silking Interval | 0.432* | 0.434* | −0.038 | −0.094 |
| 50% silking (Days) | 0.105 | 0.561** | −0.007 | −0.398 |
| 50% pollen (Days) | 0.262 | 0.613 | −0.01 | −0.400* |
| Grain filling (Days) | −0.344 | −0.587** | 0.259 | 0.001 |
| Maturity (Days) | −0.222 | −0.25 | 0.253 | −0.155 |
| Plant height (cm) | 0.209 | 0.658** | 0.244 | −0.19 |
| Ear height (cm) | 0.25 | 0.663** | 0.148 | −0.091 |

1 = * Significant at $P \leq 0.05$, ** Significant at $P \leq 0.01$.

both sites, aflatoxin accumulation was positively correlated with diseased ears. The *A. flavus* and aflatoxin were significantly positively correlated with Anthesis to silking interval at Seatondale. Aflatoxin was significantly positively correlated with insect damage, husk tightness, plant height, ear height and days to 50% silking at Seatondale, but negatively correlated with grain filling days and ear droopiness. However, at Igeri site, *A. flavus* was not correlated with any variable, including aflatoxin content.

### *3.3.2. Correlation between A. flavus, aflatoxin and kernel characteristics*

Correlation coefficients between *A. flavus*, aflatoxin and kernel characteristics are presented in Table 4. At Seatondale, both *A. flavus* and aflatoxin accumulation were negatively correlated with kernel ash content. At Igeri, aflatoxin accumulation was not correlated with any kernel characteristics. However, *A. flavus* was highly significantly positively correlated with kernel moisture and kernel ash content.

## 4. Discussion

The application of N and P fertilizer rates did not cause significant differences in *A. flavus* and aflatoxin development in maize, partly because the environmental condition during the conduct of trials as well as the use of needless syringe inoculation technique, might have contributed to the relatively low aflatoxin contamination noted in this study. Additionally, the split plot design used usually sacrifices precision in estimating the average effects of the treatments assigned to main plots. However, this study revealed significant variations in levels of *A. flavus* and aflatoxin in maize grains from Seatondale and Igeri, with higher levels at Seatondale than at Igeri. The differences noted could be attributed to contrasting weather conditions at the two sites. Seatondale had higher mean temperatures and lower rainfall distribution during the trial season, which may have favoured *A. flavus* to produce more aflatoxin compared to the Igeri site. Hence, this discussion concentrates mostly on differences noted among the hybrids in terms of plant

**Table 4**
Correlation coefficients between *A. flavus*, Aflatoxin and kernel characteristics for Seatondale and Igeri sites.

| Kernel variables | Site | | | |
|---|---|---|---|---|
| | Seatondale | | Igeri | |
| | *A. flavus* | Aflatoxin (μg kg$^{-1}$) | *A. flavus* | Aflatoxin (μg kg$^{-1}$) |
| Aflatoxin (μgkg$^{-1}$) | 0.606** | – | 0.051 | – |
| Grain yield (t/ha) | 0.097 | 0.256 | 0.297 | −0.19 |
| Dry Matter (%) | 0.226 | 0.243 | −0.085 | 0.006 |
| Kernel moisture (%) | −0.226 | −0.243 | 0.677** | 0.255 |
| Kernel density (g/mL) | −0.039 | −0.132 | −0.232 | 0.076 |
| Kernel texture (score) | 0.284 | 0.211 | 0.085 | −0.006 |
| Kernel ash content | −0.468** | −0.550** | 0.181**[a] | −0.015 |

**Significant at $P \leq 0.01$.
[a] Significant based on transformed data.

and kernel characteristics as well as *A. flavus* and aflatoxin accumulation in the grains based mostly on results from Seatondale.

Despite the insignificant effect of application of N and P fertilizer rates, there was a marked trend in the reduction of aflatoxin levels with increased N and P application. For instance, a 35.65% reduction in aflatoxin level was observed when optimal N and P rates ($N_1P_1$) were applied at Seatondale. The mean aflatoxin concentration for the optimal rate ($N_1P_1$) and sub optimal ($N_2P_2$) was 4.77 μg kg$^{-1}$ and 8.67 μg kg$^{-1}$, respectively. Previous studies are in support of these findings. For instance, studies in North Carolina State showed that N stressed corn had 28% more aflatoxin contamination in wound inoculated ears which received suboptimal N than ears that received optimal N (Payne et al., 1989). The application of optimal combinations of N and P often speed up the growth of maize seedlings and roots, hence improving crop health and creating strong cell walls which may act as mechanical barriers against the entry of *A. flavus* (Hubber and Haneklaus, 2007; Zhao et al., 2009).

Generally, all the six hybrids were found to be susceptible to *A. flavus* and aflatoxin accumulation. However, the susceptibility varied among the hybrids and was mostly associated with variables such as ear droopiness, husk tightness, days to 50% silking and 50% pollen shed, anthesis to silking interval, diseased ears, insect damaged ears, kernel texture, grain filling, ear height, kernel ash content and kernel moisture content according to simple linear correlation analyses. Some of these variables were also noted by previous researchers (Warburton and Williams, 2014). Aflatoxin contaminations were found to be low in those hybrids having completely drooped ears. Maize ears start drooping at physiological maturity and the extent of droopiness depends on the genotype. When the ears droop, they prevent moisture from the condensed air or off seasonal rain getting into the kernels through the ear tips, especially for hybrids with tight husk covers and closed ear tips. Ear height was negatively correlated with aflatoxin, where hybrids with short ear heights were found to have high aflatoxin. The maize ears placed closer to the soil surface are known to have possibilities of picking extra *A. flavus* inoculum from the soil as most of the *A. flavus* populations live in the soils as saprophytes (Payne et al., 1989).

The present study revealed that prolonged period of flowering increased the chance of aflatoxin development in grains. The concentration of aflatoxin in maize was found to be relatively higher in those hybrids that had delayed anthesis. This implies that, as the silk channels become viable for a relatively long period before pollination, the risk of the plant to pick spores of *A. flavus* from the surrounding increases given that silk channels are one of the known entry points of *A. flavus* into maize kernels (Windham et al., 2000). The association between prolonged pollination and increased levels of aflatoxin in maize has also been reported previously by Betránd and Isakeit (2004). Wounds on grains and cobs caused by insects during their feeding remove natural barriers that protect maize grains and may increase the area accessible for *A. flavus* colonization (Cleveland et al., 2003; Warburton and Williams, 2014).

Hybrid UHS 5210 accumulated the lowest aflatoxin content. This hybrid possesses characteristics that might have prevented the entry and development of *A. flavus*. The hybrid had many husk leaves, flint kernels, very tight husk covers and few open ear tips. A tight husk and non-upright ear prevent entry of spores and keep the ear dryer, making it a less conducive environment for fungal growth (Dean Barry et al., 1986). Some traits, such as open tips, were not significantly correlated with *A. flavus* and aflatoxin; however, these traits were significantly correlated with other predisposing traits such as insect damage that is known to enhance *A. flavus* entry and development.

Although the amount of *A. flavus* in grains in this study seems to be relatively low, such low levels in farmers' maize may serve as initial inoculum that may increase at post-harvest if the conditions at storage and or during transportation favours their multiplication. The challenges of developing and using host plant resistance for reduction of aflatoxin problem have recently been reviewed by Warburton and Williams (2014). As such, any percentage reduction of aflatoxin concentration caused by proper use of host resistance and field management is crucial to public health and trade of maize food and feeds.

## 5. Conclusion

Despite the insignificant influence of N and P fertilizer combinations on Aflatoxin accumulation noted in this study, the trend demonstrated by the application of optimum rates indicate that N and P fertilizer rates could be fine-tuned to mitigate the problem of aflatoxin contamination in maize kernels at pre harvest stage as. Furthermore, despite the susceptibility to aflatoxin contamination reported in most of the commercially available maize hybrids in southern highlands of Tanzania, a few varieties, notably Hybrid UHS 5210, had relatively low susceptibility to aflatoxin accumulation and could be included as a component in the development of integrated management of *A. flavus* and aflatoxin accumulation at preharvest stage.

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## References

Abbas, H.K., Weaver, M.A., Horn, B.W., Carbone, I., Monacell, J.T., Shier, W.T., 2011. Selection of *Aspergillus flavus* isolates for biological control of aflatoxins in corn. Toxin Rev. 30 (2–3), 59–70.

Aflacontrol Project, Note 1, 2010. Reducing the Spread of Aflatoxins in Kenya and Mali. International Food Policy Research Institute. http://programs.ifpri.org/afla/afla.asp. Last visited on 10.11.2013.

Atehnkeng, J., Ojiambo, P.S., Donner, M.T., Sikora, R.A., Cotty, P.J., Bandyopadhyay, R., 2008. Distribution and toxigenicity of *Aspergillus* species isolated from maize kernels from three agro-ecological zones in Nigeria. Int. J. Food Microbiol. 122, 74–84.

Betránd, F.J., Isakeit, T., 2004. Aflatoxin accumulation in maize hybrids of different maturities. J. Agron. 96, 565–570.

Bisanda, S., Mwangi, W.M., Verkuijl, H., Moshi, A.J., Anandajayasekeram, P., 1998. Adoption of Maize Production Technologies in the Southern Highlands of Tanzania. CIMMYT/The United Republic of Tanzania/SACCAR, Mexico, DF (Mexico), p. 38.

Brown, L.R., Zhi-Yuan, C., Abebe, M., Thomas, E.C., 2003. Using Biotechnology to enhance Host resistance to aflatoxin contamination of corn. Afr. J. Biotech. 2 (12), 557–562.

Cardwell, K.F., Henry, S.H., 2004. Risk of exposure to and mitigation of effect of aflatoxin on human health: a West African example. J. Toxicol. -Toxin Rev. 23, 217–247.

CIMMYT, 2005. In: Laboratory Protocols: CIMMYT Applied Molecular Genetics Laboratory, third ed. CIMMYT, Mexico, D.F.

Clements, M.J., White, D.G., 2004. Identifying sources of resistance to aflatoxin and fumonisin contamination in corn grain. J. Toxicol-Toxin Rev. 23, 381–396.

Cleveland, T.E., Dowd, P.F., Desjardins, A.E., Bhatnagar, D., Cotty, P.J., 2003. U. S. Dep. Agriculture—Agricultural Res. Serv. Res. pre-harvest Prev. mycotoxins mycotoxigenic fungi U. S. crops Special Issue Pest Manag. Res. USDA Agric. Res. Serv. 59 (6–7), 629–642.

Cotty, P.J., Jaime-Garcia, R., 2007. Influences of climate on aflatoxin producing fungi and aflatoxin contamination. Int. J. Food Microbiol. 119, 109–115.

Dean Barry, E.B., Lillehoj, N.W., Widstrom, W.W., McMillan, M.S., Zuber, W.F., Kwolek Guthrie, W.D., 1986. Effect of husk tightness and insect (Lepidoptera) infestation on aflatoxin contamination of preharvest maize. Environ. Entomol. 15 (6), 1116–1118.

Diener, U.L., Davis, N.D., 1987. Biology of *A. flavus* and *A. parasiticus*. In: Zuber, M.S., Lillehoj, E.B., Renfro, B.L. (Eds.), A Proceeding of the Workshop: Aflatoxin in Maize. CIMMYT, Mexico City, pp. 33–40.

Hell, K., Fandohan, P., Bandyopadhyay, R., Kiewnick, S., Sikora, R., Cotty, J.P., 2008. Pre- and postharvest management of aflatoxin in maize. An African perspective. In: Leslie, J.F., Bandyopadhyay, R., Visconti, A. (Eds.), Mycotoxins: Detection Methods, Management, Public Health, and Agricultural Trade. CABI, pp. 219–230.

Hell, K., Mutegi, C., Fandohan, P., 2010. Aflatoxin control and prevention strategies in maize for sub-Saharan Africa. 10th international working conference on stored product protection. Julius-Kühn-Archiv 425, 534–541.

Hubber, D.M., Haneklaus, S., 2007. Managing nutrition to control plant disease. Landbauforsch. Volkenrode 57 (4), 313–322.

Kaaya, A.N., Kyamuhangire, W., 2006. The effect of storage time and agro ecological zone on mould incidence and aflatoxin contamination of maize from traders in Uganda. Int. J. Food Microbiol. 110, 217–223.

Lillohoj, E.B., Zuber, M.S., 1975. Aflatoxin problem in Corn and Possible solutions. In: Proceedings 30th Annual Corn and Sorghum Research Conference. pp. 230–250.

Lyimo, N.G., 2006. Improving Farmer's Access to and Management of Disease Resistant Cultivars in the Southern Highlands of Tanzania. Final technical Report (Unpublished). Uyole Agricultural Research Institute, Mbeya, Tanzania.

Mehan, K., McDonald, D., Ramakrishna, N., 1986. Varietal Resistance in Peanut to Aflatoxin Production. Groundnut Improvement Program. International Crops Research Institute for the Semi-Arid Tropics (ICRISAT).

Mideros, S.X., Windham, G.L., Williams, W.P., Nelson, R.J., 2009. *Aspergillus flavus* biomass in maize estimated by quantitative real-time polymerase chain reaction is strongly correlated with aflatoxin concentration. Plant Dis. 93, 1163–1170.

Miller, J.D., 2008. Mycotoxins in small grains and maize: old problems, new challenges. Food Addit. Contam. Part A 25, 219–230.

Mushongi, A.A., 2010. Genetic Studies of Secondary Traits and Yield Heterosis in Maize under Low Nitrogen Conditions Incorporating Farmers Perceptions and Preferences for Varieties (Ph.D. Thesis), pp. 54–56.

Ngasongwa, J., 2007. Tanzania Ministry of Planning and Empowerment: IRINGA (United Republic of Tanzania. Unpublished report).

Okalebo, J.R., Gathua, K.W., Woomer, P.L., 1993. Laboratory Methods of Soil Analysis: a Working Manual. Tropical Soil Biology and Fertility Programme, Nairobi, pp. 22–27.

Payne, G.A., Kamprath, E.J., Adkins, C.R., 1989. Increased Aflatoxin contamination in Nitrogen stressed corn. Plant Dis. 73, 556–559.

URT (United Republic of Tanzania), 2008. Agricultural Sector Development Programme. Government programme document. Unpublished report.

Warburton, M.L., Williams, W.P., 2014. Aflatoxin resistance in maize: what have we learned lately? Adv. Bot. Vol. 2014, 1–10.

Williams, W.P., Alpe, M.N., Windham, G.L., Ozkan, S., Mylroie, J.E., 2013. Comparison of two inoculation methods for evaluating maize resistance to *Aspergillus flavus* infection and aflatoxins accumulation. Int. J. Agron. 2013. Article ID 972316, 6 pages, http://dx.doi.org/10.1155/2013/972316.

Windham, G.L., Williams, W.P., Bren, H., Hwakins, L., 2000. Resistance of Corn to *Aspergillus flavus* Kernel Infection and Aflatoxin Contamination. Eleventh Annual National Conservation Systems, Cotton & Rice Conference Proceedings Book: p.495.

Yan-ni, Y., Lei-yan, Y., Jin-hua, J., 2008. Biological control of aflatoxin contamination of crops. J. Zhejiang Univ. Sci. B vol. 9 (10), 787. http://citations.springer.com/item?doi=10.1631/jzus.B0860003. Last visited in December, 2015.

Zhao, Q., De-Hui, Z., Zhi-Ping, F., Zhan-Yuan, Y., Ya-Lin, H., Jianwei, Z., 2009. Seasonal variations in phosphorus fractions in semi-arid sandy soils under different vegetation types. For. Ecol. Manag. 258, 1376–1382.