

Research Application Summary

Population of *Aspergillus flavus* and *Fusarium verticillioides* and contamination of maize with associated mycotoxins under push-pull cropping system in Siaya County, Western Kenya

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Abstract

Push-pull cropping system is a companion cropping system that effectively controls stemborers, fall armyworm and Striga weed, resulting in increased yield in cereal crops like maize and sorghum. Siaya County is one of the administrative localities where push-pull cropping system has been adopted by smallholder farmers practising mixed cropping. The aim of this study was to assess the population of *Aspergillus flavus*, *Fusarium verticillioides* and levels of aflatoxin and fumonisin in maize under push-pull cropping system in Siaya County. A total of 57 maize samples i.e., 27 push-pull and 30 non-push-pull, were collected at harvest between January and February 2017. Ten to 20 maize cobs were collected from standing crop from each farm. Fungi were isolated from ground homogenized maize samples on low strength potato dextrose agar amended with chloramphenicol, streptomycin and tetracycline and identified based on their cultural and morphological characteristics. Levels of aflatoxin and fumonisin were determined from 20g of maize composite samples using Enzyme-Linked Immuno-Sorbent Assay (ELISA). *Fusarium*, *Aspergillus* and *Acremonium* spp. were the most prevalent fungal genera in maize samples from both push-pull and non-push-pull cropping systems. The population of *F. verticillioides* and *A. flavus* was significantly lower in maize samples from push-pull cropping system. All the samples from push-pull cropping system had aflatoxin levels below the Kenyan threshold (10 parts per billion). The proportion of maize samples with high fumonisin levels above the threshold set by European Commission (1000 ppb) was 10.6%, which was lower in maize from push-pull cropping system. The findings of this study showed that there were significantly lower populations of *A. flavus* and *F. verticillioides* and lower levels of aflatoxin and fumonisin from maize samples collected from farms practising push-pull cropping system. These findings imply possibility of a mechanism of control of mycotoxigenic fungi and associated mycotoxins by the push-pull cropping system. The high population of *F. verticillioides* in maize was an indicator of the risk of further fumonisin production in storage and hence exposure of the toxin to humans and animals.

Key words: Aflatoxin, fumonisin, fungi, Kenya, mycotoxins, push-pull cropping system, Siaya county

Résumé

Le système de culture push-pull est un système de culture compagnon qui contrôle efficacement les foreurs de tiges, la légionnaire d'automne et les mauvaises herbes *Striga*, ce qui augmente le rendement des cultures céréalières comme le maïs et le sorgho. Le comté de Siaya est l'une des localités administratives où le système de culture push-pull a été adopté par les petits agriculteurs pratiquant la culture mixte. Le but de cette étude était d'évaluer la population d'*Aspergillus flavus*, *Fusarium verticillioides* et les niveaux d'aflatoxine et de fumonisine dans le maïs sous le système de culture push-pull dans le comté de Siaya. Au total, 57 échantillons de maïs, soit 27 push-pull et 30 non-push-pull, ont été collectés à la récolte entre janvier et février 2017. De 10 à 20 épis de maïs ont été récoltés dans les cultures sur pied de chaque ferme. Les champignons ont été isolés d'échantillons de maïs homogénéisés moulus sur une gélose au dextrose de pomme de terre à faible concentration modifiée avec du chloramphénicol, de la streptomycine et de la tétracycline et identifiés en fonction de leurs caractéristiques culturelles et morphologiques. Les niveaux d'aflatoxine et de fumonisine ont été déterminés à partir de 20 g d'échantillons composites de maïs en utilisant le dosage immuno-absorbant lié aux enzymes (ELISA). *Fusarium*, *Aspergillus* et *Acremonium* spp. étaient les genres fongiques les plus répandus dans les échantillons de maïs des systèmes de culture push-pull et non-push-pull. La population de *F. verticillioides* et *A. flavus* était significativement plus faible dans les échantillons de maïs du système de culture push-pull. Tous les échantillons du système de culture push-pull avaient des niveaux d'aflatoxine inférieurs au seuil kenyan (10 parties par milliard). La proportion d'échantillons de maïs avec des niveaux élevés de fumonisine au-dessus du seuil fixé par la Commission européenne (1000 ppb) était de 10,6%, ce qui était plus faible pour le maïs issu du système de culture push-pull. Les résultats de cette étude ont montré qu'il y avait des populations significativement plus faibles d'*A. Flavus* et *F. verticillioides* et des niveaux inférieurs d'aflatoxine et de fumonisine dans des échantillons de maïs collectés dans des exploitations pratiquant le système de culture push-pull. Ces résultats impliquent la possibilité d'un mécanisme de contrôle des champignons mycotoxigènes et des mycotoxines associées par le système de culture push-pull. La forte population de *F. verticillioides* dans le maïs était un indicateur du risque de poursuite de la production de fumonisine pendant le stockage et donc de l'exposition de la toxine aux humains et aux animaux.

Mots clés: Aflatoxine, fumonisine, champignons, Kenya, mycotoxines, système de culture push-pull, comté de Siaya

Introduction

Mycotoxins are toxic secondary metabolites that are produced by fungi during their growth (Negedu *et al.*, 2011). The amount of mycotoxin produced by a fungus is influenced by the environmental and nutritional parameters at the time of production, the previous growth history and development of the fungi. Temperature, moisture and relative humidity are the most important ecological factors that influence mycotoxin contamination of grains (Fountain *et al.*, 2014). Maize contaminated with unacceptable levels of mycotoxins poses great economic losses and risk to agricultural trade (Leslie *et al.*, 2005). The most common mycotoxins of maize are aflatoxins and Fumonisin (Kimanya, 2015). Aflatoxins are

produced by members of *Aspergillus* section Flavi (Samson and Varga, 2009). *Aspergillus flavus* and *A. parasiticus* are the most important producers of aflatoxin in food with maize and peanuts being the most susceptible to contamination with the toxin (Samson and Varga, 2009; Ezekiel *et al.*, 2014). There are four main types of aflatoxins, aflatoxin B1, B2, G1 and G2. *Aspergillus flavus* mainly produces aflatoxin B1 and B2 while *A. parasiticus* produces all the four types of aflatoxins. Consumption of aflatoxin may cause liver damage, cancer, retards growth of children, suppresses the immune system in humans and decreases productivity in humans and animals. Acute exposure to high aflatoxin levels can also be fatal (Lewis *et al.*, 2005).

Fumonisin is a mycotoxin that is mainly produced by *F. verticillioides*, and to a lesser extent by *F. proliferatum* (Coulibaly *et al.*, 2008). The fungi associated with fumonisin cause Fusarium ear rot in maize, whose infection is increased by physical damage to kernels by insects feeding on the cob. Consumption of cereals contaminated with fumonisin causes clinical symptoms such as apoptosis of the liver and kidneys, pulmonary edema, esophageal cancer, neural tube birth defects and toxification of the nervous system (Olga, 2009). Drying the grains below 14% moisture content stops fumonisin production in storage, but fumonisin produced before harvest and drying will remain intact (Ono *et al.*, 2002).

Maize contaminated with unacceptable levels of mycotoxins poses great economic losses and risk to agricultural trade because contaminated cereals are not accepted into international markets and when detected, the cereals are destroyed (Coulibaly *et al.*, 2008). It is estimated that about 40% of grain losses in Sub Saharan Africa are due to mycotoxins. There are legal upper limits/thresholds for major mycotoxins maize intended for human consumption by various organizations; Kenya Bureau of Standards (KEBS), European Union (EU), Food and Drug Administration (FDA), on the maximum of each mycotoxin acceptable in food and feed.. European Commission (EC) regulation has set maximum acceptable levels of aflatoxin and fumonisin at 4ppb and 1000ppb, respectively, (EC R1881, 2006) while United States Food and Drug Agency (FDA) has set threshold for the two mycotoxins at 20ppb and 4000ppb, respectively (National Grains and Feed Association, 2011). Kenya Bureau of Standards has threshold only for aflatoxin, 10ppb, therefore adopts the EC fumonisin threshold.

Push-pull cropping system is a companion cropping system that involves intercropping cereals with insect repellent crops ('push') and planting attractive trap plants ('pull') around this intercrop. The cropping system effectively controls parasitic weed *Striga* and lepidopteran pests, such as stemborers and fall armyworm (FAW), resulting in improved crop yields (Khan *et al.*, 2010; Midega *et al.*, 2010 and 2018). The intercrops, principally *Desmodium* emit repellent chemicals that 'push' away the insects that are simultaneously attracted to the trap plants that emit attractive volatiles. *Desmodium* spp. also suppress the parasitic *Striga* weed and improve soil health through a number of mechanisms including nitrogen fixation and improved organic matter content and conservation of soil moisture. The companion crops are valuable fodder thereby allowing crop-livestock integration. The cropping system has been adopted by over 150,000 small holder farmers in eastern Africa (<http://www.push-pull.net/adoption.shtml>).

Materials and Methods

Sampling, isolation and identification of *Aspergillus* spp. and *Fusarium* spp. from maize samples. Field sampling was conducted in Ugunja Sub-County in Siaya County between January

and February 2017. Ten to 20 maize cobs were collected by randomly picking from the standing crop from 57 farms: 27 push-pull and 30 non-push-pull. The cobs were sub-dried for two weeks, shelled and then finely ground using a coffee mill. Isolation of fungi was done on low strength potato dextrose agar (PDA – 17g, KH_2PO_4 – 1g, KNO_3 – 1g; MgSO_4 – 0.5g, agar - 10g). The media was amended with 50 mg of tetracycline, streptomycin and chloramphenicol. One-gram maize sub-sample was suspended in 9 ml of sterile distilled water, vortexed for 30 seconds and appropriately serially diluted. One hundred microliter aliquots were spread on the media in triplicate with sterile bent glass rods. After incubation at 25°C for 3 days, fungal colonies were identified, counted and sub-cultured with sterile toothpicks to PDA and incubated at 25°C for 7-14 days. Colonies of *Fusarium* spp. were also sub-cultured on synthetic nutrient agar (SNA: Nirenberg, 1981; KH_2PO_4 1.0g, KNO_3 1.0g, MgSO_4 0.5g, KCl 0.5g, Glucose 0.2g, Agar 20g) and incubated for 14-21 days at near UV light while colonies of *Aspergillus* spp. were sub-cultured on Czapek Dox agar and incubated for 3 – 5 days. The number of colony forming units per gram (CFU/g) of ground maize was calculated using the formula:

$$\text{CFU/g soil} = \frac{\text{No. of colonies}}{\text{Amount plated} \times \text{Dilution}}$$

The percentage occurrence of different fungal genera was calculated as:

$$\text{Frequency (\%)} = \frac{\text{Number of isolates of a genus}}{\text{Total number of genera}} \times 100$$

Fungal genera were identified based on their cultural and morphological characteristics using the manual by Humber (1997). *Fusarium* spp. were identified using manuals by Nelson *et al.* (1983) and Leslie and Summerell (2006) while *Aspergillus* spp. were identified using the manual by Klich (2007). Colonies of *Fusarium* spp. sub-cultured on SNA were used for microscopic identification while those transferred to PDA were used for cultural characterization.

Detection and quantification of aflatoxin and fumonisin levels in maize samples. Twenty grams sub-samples were weighed in duplicate and extracted with 100 ml of 70% methanol and 40 ml of 90% methanol for aflatoxin and fumonisin analyses, respectively, by shaking. The particulate matter was allowed to settle, the extracts filtered through Whatman no.1 filter paper and the filtrate collected for testing. The sample extracts for fumonisin analysis were diluted with distilled water in the ratio of 1:20. Aflatoxin and fumonisin levels were quantified by direct competitive Enzyme Linked Immuno-Sorbent Assay (ELISA) (Helica Biosystems Inc.) following manufacturer's instructions. The lower and the upper limits of detection for the aflatoxin kit were 1.0 and 20.0 part per billion (ppb), respectively, while the corresponding limits of detection for the fumonisin kit were 100 and 6000 ppb, respectively. A calibration curve for the standards for each toxin was plotted and used to compare the optical densities of the samples with those of the kit standards to determine interpretative results. Samples with toxin levels above the limit of detection of the kits were diluted and the toxin levels

quantified again, with consideration of the additional dilution factor in the interpretation of the results.

Data analyses. Means and statistical differences of *Aspergillus* spp., *Fusarium* spp. and other fungal genera were calculated using Analysis of variance (ANOVA) in R software. *Aflatoxin* and *Fumonisin* data were expressed as ranges using cross tabulation procedure of SPSS version 22 within thresholds set by the Kenya Bureau of Standards (KEBS), European Commission (EC) and Food and Drug Authority of the United States of America. Data that were not normally distributed were first transformed before analyses. Spearman correlation was performed between levels of aflatoxin and fumonisin; and the population of *A. flavus* and *F. verticillioides*, respectively.

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Results and Discussion

The average fungal population (CFU/g) was 3,016.7 and 57,908.7 in maize samples from push-pull and non-push-pull farms, respectively. *Fusarium* spp., particularly *F. verticillioides*, was the main fungus isolated in samples from both cropping systems (Table 1). *Penicillium* and *Verticillium* spp. were among fungal genera isolated in low populations and frequencies. The population of *F. verticillioides* and *A. flavus* were significantly lower ($P \leq 0.05$) in samples from push-pull farms. Previous studies also reported *F. verticillioides* as the pre-dominant fungi in maize samples (Alakonya *et al.*, 2009). The high population of *F. verticillioides* in maize from non-push-pull plots was an indicator of risk of further exposure of maize to fumonisin and other *Fusarium* mycotoxins such as deoxynivalenol and zearalenone in storage. The results also imply that maize from push-pull farms is less likely to be exposed to aflatoxin production while in storage because it has less fungal population (cfu/g) at harvest.

Table 1. Fungal population (CFU/g) in maize from push-pull and non-push-pull farms in Siaya County, western Kenya

CS	FUS	ASP	AC	Others	FV	FP	FSUB	AF	AN
ppt	1,097.2	59.4	900.9	20.1	971.0	0.0	126.1	25.2	5.6
n-ppt	52,430.9	2,563.3	2539.3	19.4	52,429.3	1.2	0.0	1,039.9	1,495.0
Grand total	10467.7	787.8	1494.6	409.3	10332.3	7.3	69.7	158.0	208.9

CS – cropping system, PPT – push-pull technology, N-PPT – no push-pull technology, Asp – *Aspergillus*, Fus – *Fusarium*, Acre – *Acremonium*, Others – other fungi, FV – *F. verticillioides*, FSUB – *F. subglutinans*, AF – *A. flavus*, AN – *A. niger*

Only 11% and 25% of maize samples from push-pull and non-push-pull farms, respectively had detectable levels of aflatoxin (Table 2). The proportion of maize samples with detectable aflatoxin levels was much lower in samples from push-pull farms. Aflatoxin levels ranged from below limit of detection to 1.2 ppb in push-pull maize samples and below limit of detection to 164.9 in non-push-pull maize samples. All the maize samples from push-pull farms met the Kenyan aflatoxin threshold (10ppb), but 7% of maize samples from non-push-pull farms had aflatoxin levels above 10ppb. The levels of aflatoxin detected were significantly positively correlated (0.295^* , $P \leq 0.05$) to the population of *A. flavus* recovered from the samples.

Table 2. Concentration (ppb) of aflatoxin and proportion (%) of maize samples with various aflatoxin levels (ppb) in maize from push-pull and non-push-pull farms in Siaya County, western Kenya

CS	<LOD	1-4	>4-10	>10
ppt	88.9	11.1	0.0	0.0
n-ppt	75.0	17.9	0.0	7.1
Total	69.1	27.3	0.0	3.6

CS – cropping system, PPT – push-pull technology, N-PPT – no push-pull technology, <LOD – below limit of detection

Approximately 11% and 29% of maize samples from push-pull and non-push-pull farms, respectively had detectable levels of fumonisin (Table 3). Like the case of aflatoxin, the proportion of samples with detectable fumonisin levels was much lower, less than half, in samples from push-pull farms. Fumonisin levels ranged from below limit of detection in both cropping systems to 1337 and 9926 ppb in maize samples from push-pull and non-push-pull farms, respectively. The proportion of maize samples with fumonisin levels above the European Commission threshold (1000pb) was 11% higher for samples from non-push-pull farms. There was a significant positive correlation (0.624**, $P \leq 0.01$) between fumonisin levels and population of *F. verticillioides*. The levels of fumonisin were therefore strongly determined by the population of *F. verticillioides*.

Table 3. Concentration (ppb) of aflatoxin and proportion (%) of maize samples with various aflatoxin levels (ppb) in maize from push-pull and non-push-pull farms in Siaya County, western Kenya

CS	<LOD	100 – 1000	>1000
ppt	88.9	7.1	3.7
n-ppt	71.4	14.3	14.3
Total	80.3	12.2	9.1

CS – cropping system, PPT – push-pull technology, N-PPT – no push-pull technology, <LOD – below limit of detection

Conclusion

Maize grown under push-pull cropping system was significantly less susceptible to mycotoxigenic fungal infection and contamination with associated mycotoxins. The population of *A. flavus* and *F. verticillioides* in maize samples can be used as an indicator of the extent of aflatoxin and fumonisin contamination, respectively.

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