

Displacement of toxigenic *Fusarium* species by atoxigenic *Aspergillus flavus* (Aflasafe KE01) application in maize fields in lower Eastern Kenya

Ndirangu Esther Wanjiru^{1*}, Muthomi James², Wagacha John Maina¹ and Mutegi Charity³

¹School of Biological Sciences, University of Nairobi. P. O. Box 30197-00100, Nairobi, Kenya

²Department of Crop Science and Crop Protection, University of Nairobi. P. O. Box 30197-00100, Nairobi, Kenya

³International Institute of Tropical Agriculture. P. O. Box 30709-00100, Nairobi, Kenya

*Corresponding author's email: endirangu@kephis.org

Abstract

Maize is grown in areas with environmental conditions that are ideal for growth of most cereal pathogens such as *Fusarium* fungi. However, there is lack of effective fungicides to control fumonisin producing Fusarium. This study was conducted to determine the efficacy of atoxigenic Aspergillus flavus (Aflasafe KE01) on the population of *Fusarium* species in maize fields. The study was carried out in four sub counties in lower Eastern Kenya (Kaiti, Kathiani, Nzambani and Wote). Twenty-four maize fields were selected in each sub county where 12 fields were treated with Aflasafe KE01, while 12 fields comprised the untreated controls. Aflasafe KE01 was applied by hand broadcasting in the maize fields two to three weeks before tasselling of maize. Maize grain samples were collected from each field at harvest and ground using a Bunn coffee mill grinder. Fusarium species were isolated from the ground maize using pour plate method following serial dilution on low strength potato dextrose agar (PDA)and Spezieller Nahrstoffarmer. The results showed that application of Aflasafe KE01 effectively displaced toxigenic *Fusarium* species in maize fields. Maize samples from Aflasafe KE01 treated maize fields recorded significantly lower incidence (41%) of the *Fusarium* species compared to untreated maize fields (60%) ($p \le 0.01$). These results indicate that Aflasafe KE01 is a potential biopesticide for the biocontrol of *Fusarium* species in maize.

Key words: Aflasafe KE01, atoxigenic *Aspergillus* sp, *Fusarium* species, maize

Introduction

Maize (Zea mays L.) is an important crop to majority of Kenyan population and is grown in different agroecological zones characterized with varying temperature and rainfall (Ureta et al., 2013). Over 90% of the Kenyan population depends on maize for food (Kirimi et al., 2011; USDA, 2016). Over 38% of farmers in Kenya grow maize (FAO, 2016) and of this, small-scale farmers produce about 70% of the production. However, they overall retain up to about 58% of their total production for household consumption (Olwande *et al.*, 2015).

Maize is prone to degradation by mycotoxigenic fungi such as Aspergillus Penicillium species species, and Fusarium species which are always present in soils (Kumar et al., 2016; Koskei et al., 2020). Maize is grown in areas with environmental conditions that are ideal for growth of most cereal pathogens such as *Fusarium* fungi most of which grow in temperatures around 25-35°C and high humidity (Aldarsgarcía et al., 2018). Mycotoxins are produced by fungi in products such as maize, wheat and groundnuts which are susceptible to mould infection

(Wagacha & Muthomi, 2008; Cinar & Onbasi, 2019).

Fusarium verticillioides and Moniliforme have been identified in Kenya and are predominant in Makueni, Nandi, and western Kenya (Mutiga et al., 2015; Kang'ethe *et al.*, 2017). These regions also record high incidences of Aspergillus, Fusarium and Penicillium species (Bii et al., 2012; Kilonzi et al., 2014). A timely fungicide application reduces infection and mycotoxin contamination. However, there is lack of effective fungicides to control fumonisin-producing Fusarium. According to a survey done in Eastern and western Kenya, Fusarium species are common contaminants of maize in these areas (Koskei et al., 2020). The climatic conditions in lower Eastern region of Kenya are ideal for growth of the afore-mentioned pathogens. This has necessitated need to identify appropriate methods of controlling mycotoxins in maize and other crops. To effectively prevent or minimize future mycotoxin contamination and reduce long term exposure to mycotoxins such as aflatoxins, proper methods of controlling and reducing mycotoxin contamination are required.

This has necessitated research for alternative ways of mycotoxin management. Some of these mycotoxins can be controlled biologically through competitive exclusion methods. This is made possible by the presence of two distinct A. flavus populations: the toxigenic strains and the atoxigenic strains. Atoxigenic strains of A. flavus can effectively eradicate the highly toxigenic strains thereby reducing aflatoxin contamination. The objective of this study was to assess the effectiveness of atoxigenic A. flavus (Aflasafe KE01) in the displacement of Fusarium verticilliloides and fumonisins. This will contribute towards better management of Fusarium species in order to reduce maize degradation and mycotoxin contamination.

Materials and methods

Determination of population of *Fusarium* species in soil and maize grains in atoxigenic *Aspergillus flavus* (Aflasafe KE01) treated and untreated maize fields

Description of the study area

The study was conducted in Nzambani sub-county (Kitui County), Kathiani sub-county (Machakos County) and Wote and Kaiti sub-counties (Makueni County) in lower Eastern parts of Kenya (latitude between 4°N to 4°S, longitude 34° to 41°E). These regions receive an average rainfall of between 150 mm to 650 mm p.a. On average, Machakos and Kitui counties receive 500 to 700mm p.a and 500 to 1050 mm p.a. respectively. The soils in these areas are sandy to loamy sand texture with low organic matter contents, low water retaining capacity and low plant nutrients thus making is susceptible to (Gachimbi *et al.*, 2007). erosion Makueni county has several agroecological zones (AEZs) with altitudes from 790-1770masl ranging and receives about 600-1050mm of average annual rainfall (Jaetzold et al., 2010).

ExperimentaldesignandapplicationofatoxigenicAspergillus flavus(Aflasafe KE01)

Selection of the study sites was purposive and based on areas where maize is commonly cultivated, have a history of aflatoxin contamination and have low risk of maize crop failure (Muthomi *et al.*, 2009). The farms were selected randomly within each sub county with each farm having a minimum of 2 acres except where it

was not possible to get such farms in a particular sub county.

The experiment was conducted on maize planted by the farmers who consented to take part in the study. In each of the four sub counties, 24 maize fields were selected; 12 fields were treated with Aflasafe KE01 while the other 12 were control fields. Within each area, control fields were a maximum of 100m from the treated fields. Aflasafe KE01 was obtained from the International Institute of Tropical Agriculture (IITA). Six of the individual farmers' fields were treated with Aflasafe KE01 at an application rate of 5 kg/Ha while the other six was treated with 10 kg/Ha. Aflasafe KE01 was broadcast by hand in the selected fields 2-3 weeks prior to tasselling of maize. The experiment was carried out in one maize cropping season across the four sub counties. Data collected from the experiment included the population of *Fusarium* species in the soil and grain samples and the fumonisin levels in the maize grains.

Collection of soil and maize cob samples

Soil samples and maize cobs were sampled from experimental maize fields

to determine incidence of Fusarium species in the soil and maize grains. Additionally, maize cobs sampled were used to quantify the amount of fumonisin present in the grains. Samples of soil were collected from the farms to a maximum depth of 2cm using a spoon 2 to 3 weeks before flowering of maize iust before application of Aflasafe KE01 and from control fields. A minimum of eight maize cobs were sampled at harvest from each farm. The cobs were from randomly picked the farm following a zigzag pattern and put in bags. The bags were properly labeled with farmer details and sampling date and transported in a cool box to the laboratory for analysis within 48 hours after collection.

Soil lumps were crushed gently using a hammer and plant debris removed by hand. The soil was then sieved through sieve number 20, standard testing sieves (0.833mm opening) and air dried at 23±3°C. The maize cobs were sun dried on plastic canvas to avoid contact with the ground. Thereafter, dry maize was manually shelled by hand and dried in an oven at 45°C for two days and then crushed to fine powder with a

Bunn coffee mill grinder (Bunn omatic Corporation, Spring Field Illnois, USA). The ground maize sample was thoroughly mixed and used for isolation and identification of mycotoxigenic fungal species. The samples were stored in a refrigerator at 4 °C.

Isolation of *Fusarium* species from soil and maize kernels

Species of Fusarium were isolated from the ground maize and soil samples using serial dilution method and plating on low strength potato dextrose agar (PDA) amended with mineral salts and antimicrobial agents as described by Muthomi (2001). One gram of each soil and ground maize samples was weighed and emptied into 10ml of sterile distilled water in a 40ml glass vial. The vials were placed on vortex mixer (Velp Scientifica, Europe) for three minutes at 1750 rpm. One milliliter of the mixture was drawn using a micropipette and mixed with 9ml to form the first dilution of 10⁻¹. One milliliter of the first dilution was measured and dissolved in 9ml of sterile distilled water to form the second dilution of 10^{-2} . This was done up to the third dilution of 10⁻³. One milliliter aliquot of the second and third dilutions

(10⁻² and 10⁻³) of soil and ground maize samples was plated on the low strength PDA in triplicate. The plates were incubated at room temperature (23±3°C) for 5-7 days during which the soil and maize samples showing growth of a fungus was recorded and the fungus identified. Fusarium isolates from low strength PDA were subcultured on PDA and incubated at 25°C for 14 days. *Fusarium* species growing on PDA were identified based on their cultural characteristics.

Identification of *Fusarium* species

Different Fusarium species growing on low strength PDA were identified based on the cultural, morphological and biological characteristics such as and sporophores, spore septation shape, pigmentation, mycelia color and colony pigmentation. *Fusarium* species showing similar characteristics were given a specific code and sub-cultured on PDA and Spezieller Nahrstoffarmer Agar (SNA). The SNA medium was prepared as described by Nirenberg (1981) and autoclaved for 20 minutes at 121°C at a pressure of 15 p.s.i. Approximately 20ml was dispensed in 9cm petri dishes. Fusarium species subcultured on SNA were incubated under

UV light to facilitate sporulation. Fusarium species growing on PDA and SNA were identified following cultural (pigmentation), morphological (mycelia color and colony pigmentation) and biological (septation and sporophores, spore shape) characteristics. Morphological characteristics of various *Fusarium* isolates were used in identification to species level based on manuals by Nelson et al. (1983) and Leslie & Summerell (2006). The formula by Gonzalez et al. (1995) was used in determining the relative isolation frequency of each *Fusarium* genus.

Frequency(%)

= Number of samples in which a species occurred Total number of samples x 100

Microscopic identification of *Fusarium* species on SNA was based on macroconidial shape, septation, widest part of macro-conidia, length of apical cell, relative abundance of microconidia in aerial mycelium, micro-conidia in chains or heads, microconidial shape and conidiophores in aerial mycelium (Seifert and Gräfenhan, 2012). The number of colonies growing in each serial dilution was counted and from this the colony forming unit per gram (CFU/g) of soil or maize samples was determined using the following formula:

Number of *Fusarium* colonies/ml CFU/g= ______plated

Total dilution factor

Data analysis

Data on *Fusarium* species population and percent incidence level was subjected to analysis of variance (ANOVA) to determine significant difference in incidence of *Fusarium* species in Aflasafe KE01 treated and untreated maize fields. Differences between treated and untreated fields were separated using Fishers protected LSD ($p \le 0.05$). GenStat 15th edition analysis software was used for the analysis.

Results

Diversity of *Fusarium* species in soil and maize grains in atoxigenic *Aspergillus flavus* (Aflasafe KE01) treated and untreated maize fields

The main *Fusarium* species isolated from soil and maize samples in lower Eastern Kenya were *F. verticillioides*, *F. proliferatum*, *F. chlamydosporum*, *F. merismoides*, *F. semitectum* and *F. oxysporum*. *Fusarium verticillioides*

produced white aerial mycelium which was tinted with purple colour and the underside was dark purple on PDA while their microconidia were formed in chains. Fusarium oxysporum had a white aerial mycelium and sometimes had purple tinge on PDA. The basal part was creamy to tan orange in color while the underside was dark purple. It produced chlamydospores and microconidia borne on false head (Figure 1). Fusaruim verticillioides had an aerial white mycelium on PDA. It grew rapidly and was tinged with purple. When sporodochia was present it was tan to orange in colour. The undersurface was dark purple in colour. Its microconidia were formed in chains monophialides. Fusarium on avenaceum had scarce, very long, slender, more than three septate and thin-walled microconidia. On PDA, it had a dense white-tan aerial mycelium with dark brown colour. Fusarium *semitectum* had a rapid growing aerial mycelium that was tan in colour with very few microconidia. The macro conidia were borne in aerial mycelium and were spindled shaped, straight to slightly curved. Poliphialides were borne on the aerial mycelium. Fusarium graminearum in mature cultures had a

dense aerial mycelium which turned yellow and sporodochia were present in thick walled and mature culture with carmine red undersurface. Its macroconidia were distinctively septate, with distinctly foot shaped basal cell.



Figure 1. Growth of *Fusarium* species isolated from soil collected from maize fields on PDA

In all the sub counties, *Fusarium verticillioides* was the most predominant fungal pathogen isolated from maize samples. It had abundant microconidia which were single celled, oval shaped, non-septate microconidia. *Fusarium proliferatum* had abundant micro-conidia that were club shaped and with a flattened base which occurred in long chains and its basal cell

was foot shaped with branched polyphialids and monophialids. The rare species was *Fusarium chlamydosporum* which had spindle shaped microconidia which was either septate or nonseptate with macroconidia that were sickle shaped with a basal cell that was foot shaped and had abundant rough wall chlamydospores (Figure 2).

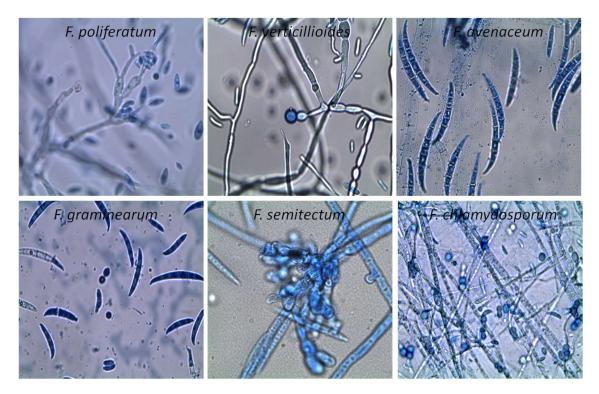


Figure 1. Various conidial types of different *Fusarium* species isolated from maize grain and soil samples from fields in lower Eastern Kenya. (x100 magnification).

Frequency of *Fusarium* species in maize sampled from Lower Eastern Kenya from atoxigenic *Aspergillus flavus* (Aflasafe KE01) treated and untreated maize fields

Incidence of the fungal pathogens varied significantly ($p \le 0.05$) in maize fields treated with Aflasafe KE01 and untreated maize fields. Maize samples from Aflasafe KE01 treated (5 kg/ha) maize fields recorded lower incidence of the *Fusarium* species compared to untreated maize fields (Figure 3a). A significant difference ($p \le 0.05$) was observed in the incidence of fungal

pathogens between maize grain samples from maize field treated with Aflasafe KE01 (10 kg/ha) and untreated maize field especially in prevalence of F. levels verticillioides, *F.* proliferatum and F. oxysporus. Aflasafe KE01-treated maize fields had significantly lower incidence of F. verticillioides, F. proliferatum and F. oxysporus compared to untreated maize fields (Figure 3b).

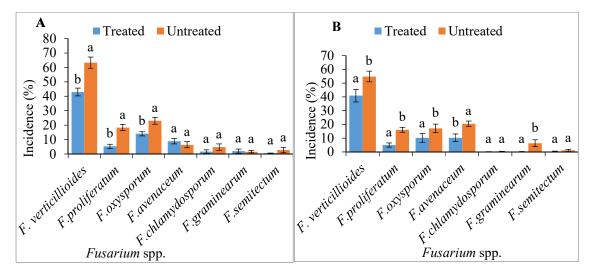


Figure 3. Frequency (%) of *Fusarium* spp. in maize grains sampled at harvest from fields treated with 5kg/ha (A) and 10kg/ha (B) and untreated maize fields. Error bars represent the standard error of the means. Means with different letters for each species are significantly different from each other at $a \le 0.05$.

The 5kg/ha rate application of Aflasafe KE01 significantly reduced incidences of F. verticillioides and F. proliferatum in Kaiti sub-county while in Kathiani subcounty there was a significant difference in the incidence of F. proliferatum and F. oxysporum between maize samples from Aflsasfe KE01 treated and untreated fields (Table 1). The incidence of Fusarium verticillioides varied significantly at ($p \le 0.05$) in treated and untreated maize fields in Kaiti and Kathiani sub-counties but there was no significant difference at ($p \le 0.05$) fields in Nzambani and Wote. Fusarium proliferatum and F. avenaceum varied significantly in maize fields from Kaiti sub-county only. There were insignificant differences in the population of F.

oxysporum, F. avenaceum, F. chlamydospora, F. graminearum and F. semitectum between treated and untreated maize fields in all the four sub counties. There was a general decrease in the level of *Fusarium* species in treated maize fields compared to untreated fields (Table 1).

Table 1. Frequency (%) of <i>Fusarium</i> spp. in maize grains sampled at harvest from fields treated with 5kg/ha and 10 kg/ha Aflasafe
KE 01 in Lower Eastern Kenya.

<i>Fusarium</i> spp.	Treatment	5kg Aflasafe KE 01				10kg Aflasafe KE 01					
		Kaiti	Kathiani	Nzambani	Wote	Mean	Kaiti	Kathiani	Nzambani	Wote	Mean
F. verticillioides	Treated	23.7b	63.2a	74.2a	57.0a	63.3	43.5a	45.2a	73.6a	54.7a	54.3
	Untreated	58.8a	44.9a	62.7a	40.9a	43.1	41.7a	23.4b	75.4a	43a	45.9
F. proliferatum	Treated	1.8b	0.6b	4.9a	13.9a	5.3	4.1b	6b	4.8a	5.2a	5
	Untreated	31.0a	16.7a	0.0a	24.8a	18.1	34a	18.2a	2.8a	9.2a	16
F. oxysporum	Treated	36.0a	4.6b	1.4a	14.5a	14.1	27.8a	20.4a	4.6a	12.1a	16.2
	Untreated	39.7a	20.2a	3.4a	28.5a	23	15.9a	4.5a	18.4a	31.4a	17.6
F. avenaceum	Treated	2.5a	22.8a	8.3a	2.2a	9	23.3a	23a	11.4a	24.3a	20.5
	Untreated	5.4a	12.8a	3.7a	3.7a	6.4	8.3b	23.2a	0a	9.5a	10.3
<i>F.</i>	Treated	0.0a	0.0a	0.0b	6.9a	1.7	0.5a	0a	0a	0a	0.1
chlamydosporum	Untreated	0.0a	0.0a	19.1a	0.0a	4.8	0.1a	0a	0a	1.3a	0.4
F. graminearum	Treated	0.0a	7.9a	0.0a	0.0a	2	0.8a	0b	0a	0a	0.2
	Untreated	0.0a	4.3a	0.0a	2.2a	1.6	0a	20.7a	3.5a	1.2a	6.4
F. semitectum	Treated	0.9a	0.9a	0.0b	0.0a	0.5	0a	0a	0a	1.7a	0.4
	Untreated	0.2a	0.0a	11.1a	0.0a	2.8	0a	0a	0a	4.4a	1.1

Means followed by the same letter(s) within columns for each species in each sub-county are not significantly different (Fisher's protected LSD at $p \le 0.05$).

Population of fungal pathogens in maize sampled from atoxigenic *Aspergillus flavus* (Aflasafe KE01) treated farms in lower Eastern Kenya

The population of fungal pathogens varied significantly ($p \le 0.05$) in maize fields treated with Aflasafe KE01 and untreated maize fields especially in F. verticillioides, F. proliferatum and F. oxysporum. Maize samples from atoxigenic *A. flavus* treated (5 kg/ha) maize fields recorded lower populations of Fusarium species compared to untreated maize fields (Figure 4a). Application of Aflasafe KE01 at the rate of 5kg/ha significantly reduced incidences of F. verticillioides and F. proliferatum in Kaiti sub-county while in Kathiani, significant differences were observed in the incidence of F. F. proliferatum and oxysporum between maize grains from treated and untreated fields (Table 2).

The population of *F. verticillioides* varied significantly at ($p \le 0.05$) in treated and untreated maize fields in Kaiti and Kathiani sub-counties. However, in Nzambani and Wote, there wERE insignificant differences ($p \le 0.05$) (Table 2). *Fusarium proliferatum* and *F.*

avenaceum varied significantly in maize fields from Kaiti sub-county only. There were insignificant differences in the population of *F. oxysporum, F. avenaceum, F. chlamydospora, F. graminearum and F. semitectum* between treated and untreated maize fields in all the four sub-counties. There was a general decrease in the level of *Fusarium* species in treated maize fields as compared to untreated fields (Table 2).

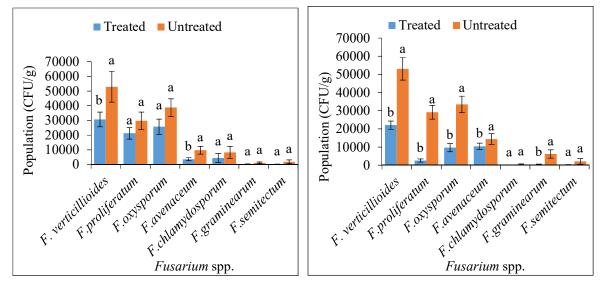
Table 2. Population (CFU/g) of fungal pathogens in maize kernels sampled from farms treated with 5kg/ ha in four sub-counties i	n
lower Eastern Kenya.	

			5 kg/ha					10 kg/ha	
Fusarium spp.	Treatment	Kaiti	Kathiani	Nzambani	Wote	Kaiti	Kathiani	Nzambani	Wote
F. verticillioides	Treated	43333a	23611b	14667a	27500a	21660a	19444b	21889a	25278b
	Untreated	57222a	66667a	59333a	37500a	73889b	44722a	33722a	59722a
F. avenaceum	Treated	556a	11389b	1389a	1111a	14167a	8889b	7500a	11111a
	Untreated	7222a	28056a	556a	3056a	10833a	31389a	0a	15278a
F. graminearum	Treated	0a	1111a	0a	0a	556a	0b	0a	1111a
	Untreated	0a	2778a	0a	1944a	0a	21111a	1389a	2222a
F. semitectum	Treated	278a	556a	0a	0a	0a	0a	0a	833a
	Untreated	278a	0a	0a	0a	0a	0a	0a	8333a
F. chlamydospora	Treated	0a	0a	0b	17778b	278a	0a	0a	0a
	Untreated	0a	0a	33229a	0a	278a	0a	0a	1667a
F. proliferatum	Treated	1944b	33056a	0b	50000a	1944b	3056a	3056a	2222b
	Untreated	99167a	278b	2222a	17500b	55833a	43333b	556a	16944a
F. oxysporum	Treated	43056a	3056b	278a	19444a	20000a	9722b	3889a	5000b
	Untreated	80278a	50556a	4722a	56667a	30278a	24444a	26667a	52500a

Means followed by the same letter(s) within columns in each sub county are not significantly different (Fisher's protected LSD at $p \le 0.05$).

There was a significant difference $(p \le 0.05)$ in the population of fungal pathogens between the maize grain sampled from fields treated with Aflasafe KE01 (10 kg/ha) and untreated maize fields especially in the population of *F. verticillioides, F. proliferatum and*

F. oxysporus. Aflasafe KE01-treated maize fields had significantly lower population of *F. verticillioides, F. proliferatum and F. oxysporum* compared to the untreated maize fields (Figure 4).



Error bars represent the standard error of the means. Means with different letters for each species are significantly different from each other at $\alpha \le 0.05$.

Figure 4. The population (CFU/g) of fungal pathogens in maize sampled from farms treated with 5 kg/ha (A) and 10kg/ ha (B) of Aflasafe KE01 in lower Eastern Kenya.

Discussion

From this study, there was high contamination of maize samples with *Fusarium* species. This may be attributed to the conducive weather conditions of negligible rainfall and high temperatures experienced by preharvest stages of maize. According to Goertz *et al.* (2010), small amounts of

rainfall accompanied by high temperature in the early stages of maize development results in increased infection. Other authors have also reported that high levels of *F. verticillioides* infection are associated with drier, warmer climates (Milani, 2013; Magan and Medina, 2016). According to Doohan *et al.*, (2003) and

Munkvold, (2003), climatic conditions have a direct effect on growth, production and dispersal of inoculum, and also an indirect effect on soil and vegetation type, which may influence saprophytic survival. From this study, it is evident that a range of Fusarium species infects maize kernels in lower Eastern Kenya. The observed widespread prevalence of different Fusarium species may indicate the possibility of contamination of maize kernels by several mycotoxins other than fumonisin (Logrieco *et al.*, 2021).

Results of this study also indicated that application of atoxigenic A. flavus led to a significant decrease in the population and incidence of F. verticillioides, F. graminearum and F. proliferatum. This implies that the atoxigenic A. flavus strains present in Aflasafe KE01 reduced the population and incidence of these *Fusarium* species. Similar work done in Brazil showed that the use of an atoxigenic A. flavus led to a reduction in the frequency of F. verticillioides (Reis et al., 2020). These results suggested a competition for substrate or space between fungi reducing the frequency of *Fusarium* species. Effective establishment of plant with atoxigenic strains could

competitively exclude fumonisinproducing strains or prevent them from producing fumonisins (Pereira et al., 2011). The action of competitive exclusion occurs when the nonaflatoxigenic strains effectively compete for space and nutrients, thus excluding their aflatoxigenic counterparts (Kagot *et al.*, 2019). The atoxigenic A. flavus strains found in Aflasafe KE01 excluded the toxigenic strain from the niche and competed for nutrients destined for fumonisin biosynthesis.

Other fungal species have been reported to have antagonistic effects that inhibit the growth and proliferation of Fusarium species (Wachowska et al., 2013; Samsudin and Magan, 2016; Abdallah *et al.*, 2018). Luongo *et al.* (2005) also reported suppression of saprophytic colonization and sporulation of toxigenic *F. verticillioides* and *F. proliferatum* in maize residues by non-pathogenic Fusarium species. Wagacha & Muthomi (2008) suggested that atoxigenic strains of F. *verticillioides* and *F. proliferatum* would be superior bio-control agents for toxigenic strains since they occupy the same ecological niche as the toxigenic

strains in the host plant and share similar growth conditions. This study played a significant role in inhibiting *Fusarium* development on maize kernels across the four sub-counties.

Conclusions

Diversity of the fungal population in lower Eastern Kenya varied among different Fusarium species with F. verticillioides beina the most predominant species isolated across the four sub-counties. Fusarium grameniearum was the least isolated Fusarium species across all the four sub-counties. Findings from this study showed that application of atoxigenic A. *flavus* (Aflasafe KE01) effectively displaced toxigenic Fusarium species in maize in lower Eastern Kenya and thus is a good candidate for a biopesticide.

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showed that the concurrent presence of *A. flavus*

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