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Field efficacy of a mixture of atoxigenic *Aspergillus flavus* Link: Fr vegetative compatibility groups in preventing aflatoxin contamination in maize (*Zea mays* L.)

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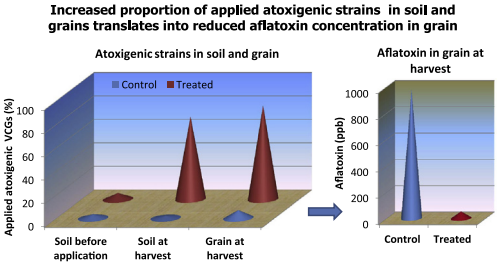
HIGHLIGHTS

- A mixture of 4 atoxigenic strains of *Aspergillus flavus* was applied in maize fields.
- Applied atoxigenic strains increased, while toxigenic strains reduced in soil and grain.
- Fewer toxigenic strains resulted in low aflatoxin at harvest and after poor storage.
- Application of strain mixture in maize fields did not increase moldiness of grain.
- Preharvest application of strain mixture can dramatically reduce aflatoxin in grain.

GRAPHICAL ABSTRACT



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ABSTRACT

Competitive exclusion of aflatoxin producers by endemic atoxigenic strains of *Aspergillus flavus* is a proven tool for aflatoxin management being adapted for use in Africa. Field efficacy of an experimental formulation consisting of four native atoxigenic strains (La3303, La3304, La3279 and Ka16127) was evaluated on maize in 2007 and 2008 in four agroecological zones in Nigeria. The four atoxigenic strains were individually formulated on sterile sorghum grain and subsequently mixed in equal proportions. The blended product was applied on soil (40 kg/ha), 2–3 weeks before flowering. Grains from treated and untreated fields were analyzed for aflatoxins at harvest and after storage. Proportions of the *A. flavus* population composed of each of the four applied strains in soil before treatment and in harvested grains were determined using vegetative compatibility analyses. Application of the strain mixture resulted in reduced aflatoxin content and significantly ($P < 0.05$) increased the combined frequencies of the vegetative compatibility groups (VCGs) of the applied strains recovered from the soil and grain. Aflatoxin reductions of 67–95% were associated with a 74–80% combined incidence of the VCGs of the four atoxigenic strains on the treated crops. The applied atoxigenic strains remained with the crop into storage and reduced postharvest increases in contamination. The results suggest that the evaluated multi-strain formulated product has potential to contribute to reduced aflatoxin contamination in Nigeria. This is the first report of a field evaluation of an endemic strain mixture effective at reducing aflatoxin contamination during crop development.

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1. Introduction

Field crops in Sub-Saharan Africa are frequently contaminated by *Aspergillus flavus* Link:Fr, *Aspergillus parasiticus* Speare and *Aspergillus nomius* Kurtzman et al. (Bandyopadhyay et al., 2007) These *Aspergillus* species infect crops both in the field and after harvest in storage, contaminating them with aflatoxins. Corn (*Zea mays* L.), peanuts (*Arachis hypogaea* L.), cottonseed (*Gossypium* species), chilies (*Capsicum annuum* L.), and various tree nuts are particularly susceptible to preharvest aflatoxin contamination when produced under high temperature and moisture stress and when insect injury is prevalent (Cotty and Jaime-Garcia, 2007). Aflatoxin exposure in humans and animals is chronic in West Africa since contamination is widespread in major food and feed sources such as maize, groundnut and yam chips (Bankole and Adebanjo, 2003; Hell et al., 2003; Kpodo and Bankole, 2008). In addition to being potent hepatotoxic and carcinogenic metabolites (Liu and Wu, 2010), aflatoxins suppress the immune system increasing susceptibility of humans to infections and also retard growth and development in young children (Jolly et al., 2008; Khlangwiset et al., 2011). Livestock and poultry are similarly affected (Diekman and Green, 1992).

Aflatoxins are closely monitored and regulated in developed countries due to health hazards in humans and productivity losses in animal industries (Van Egmond, 2002). Agricultural economies are also affected by aflatoxin contamination due to loss of produce and the time and cost involved in monitoring and decontamination efforts (Shane, 1994). Sustainable management of aflatoxin contamination in maize can prevent aflatoxin related health hazards and prevent rejection of maize products in regional and international market. Research efforts to control and manage aflatoxin contamination in agricultural crops have focused primarily on breeding and genetic engineering for crop resistance, manipulation of agronomic practices and the use of biological control. Of these management options, biological control is perhaps the most promising and effective method for sustainable management of aflatoxin contamination in maize.

Natural communities of *A. flavus* consist of individuals that vary widely in ability to produce aflatoxin. Some isolates totally lack capacity to produce aflatoxins (i.e., atoxigenic), while others produce low (<100 ng/g) to very high (>1000 ng/g) aflatoxin concentrations. Conducive conditions for fungal infection and aflatoxin production occur frequently in West Africa, where various *Aspergillus* species have high potential to produce aflatoxins and thus, pose a threat to contaminating agricultural produce (Atehnkeng et al., 2008a; Cotty and Cardwell, 1999; Diedhiou et al., 2011; Donner et al., 2009). Based on physiological, genetic and morphological characteristics, *A. flavus* can be grouped into L- and S strains (Cotty, 1994). L strain isolates produce few, large sclerotia (average >400 μm) and highly variable quantities of aflatoxins, with some strains being atoxigenic. S strain isolates produce numerous, small sclerotia (average <400 μm) and, on average, higher aflatoxin concentrations than L strain isolates (Cotty, 1989). An unnamed taxon, known as S_{BG}, which is phylogenetically divergent from but morphologically similar to the *A. flavus* S strain, produces small sclerotia and large amounts of both B- and G-aflatoxins (Cotty and Cardwell, 1999). L-, S- or S_{BG}-strain isolates can be further subdivided into vegetative compatibility groups (VCGs) that are delineated by a heterokaryon incompatibility system (Papa, 1986). VCGs evolve as clonal lineages (Grubisha and Cotty, 2010) and aflatoxin production is more similar within VCGs than between VCGs, with some VCGs being composed of only atoxigenic members (Bayman and Cotty, 1993).

The potential for biocontrol to mitigate aflatoxin contamination has been demonstrated under field conditions with a single atoxigenic strain of *A. flavus* in cotton (Cotty, 1994), peanut (Dorner and Horn, 2007) and corn (Abbas et al., 2006) in the US where two biological control products, AF36 and afla-guard®, are commercially available. Classical selection of biocontrol agents to control plant diseases has emphasized the use of single strains of the biocontrol agent (Ojiambo and Scherm, 2006). However, disease suppression is influenced by a complex of host, pathogen and environment interactions and thus, the use of multiple strains is likely to provide an ecologically sound approach to disease suppression using biocontrol (Xu et al., 2011). It has been suggested that compared to single strains, multiple strains of atoxigenic *A. flavus* could result in a synergistic activity that would lead to a greater reduction in aflatoxin contamination under field conditions (Bandyopadhyay and Cardwell, 2003; Mehl et al., 2012; Probst et al., 2011). Several thousand native isolates of *A. flavus* have been collected in Nigeria and tested for their capacity to produce aflatoxin and many atoxigenic isolates have been identified (Atehnkeng et al., 2008a, 2008b). Native atoxigenic isolates also alleviates some of the concerns on safety and environmental impacts that might be of greater concern when introduced non-indigenous microorganisms are used in biological control (Probst et al., 2011). Further, strains belonging to these naturally occurring atoxigenic VCGs do not to make aflatoxins due to defects in the aflatoxin biosynthesis gene cluster (Donner et al., 2010). However, mixtures of these strains have not been evaluated for their ability to reduce aflatoxin contamination in maize. Thus, this study was conducted to evaluate the efficacy of an experimental product containing four atoxigenic L strain isolates belonging to distinct VCGs of *A. flavus* as active ingredients in reducing aflatoxin contamination in large plots of developing maize in four agroecological zones in Nigeria. The results demonstrate that the experimental product containing multiple endemic atoxigenic strains of *A. flavus* have value in the mitigation of aflatoxin contamination.

2. Materials and methods

2.1. Fungal strains and production of strain mixture

Four indigenous atoxigenic isolates (La3303, La3304, La3279 and Ka16127) of the L strain morphotype of *A. flavus* isolated from maize grains in Nigeria (Atehnkeng et al., 2008b) were used to manufacture the experimental strain mixture used in this study. Each of these four isolates belonged to a distinct VCG whose membership is restricted only to atoxigenic isolates (J. Atehnkeng, *unpublished data*). La3303, La3304 and La3279 were obtained from maize produced in Lafia, Nassarawa State. Isolate Ka16127 was cultured from maize produced in Saminaka, Kaduna State. Conidial suspensions of the four atoxigenic strains were made from 5-day-old cultures on 5/2 agar (Cotty, 1994; 5% V8 juice, 2% agar, pH 5.2) in 0.1% Tween 80 and adjusted to 1×10^6 conidia per ml (Bock and Cotty, 1999) using a haemacytometer. To formulate the strain mixture, white sorghum grains (1 kg) were soaked in water for 2 h, drained, and autoclaved 45 min in polyethylene bags (45 × 20 cm). After cooling, grains were seeded with a conidial suspension of a single atoxigenic strain and incubated for 18 h at 31 °C to allow colonization, followed by drying at 55 °C for 4 days to inhibit further growth and sporulation. The process was repeated independently for each of the four atoxigenic strains. The end use product was formulated by combining dried grain (2.5 kg) colonized with each isolate in a polyethylene bag and mixed thoroughly by manually shaking the bags. The resultant end use experimental product containing the strain mixture was stored in 10 kg plastic containers at room temperature until use.

2.2. Field plots and inoculation

Field trials were conducted in four sites located in four agroecological zones in Nigeria; Zaria in Northern Guinea Savannah (NGS),

Mokwa in Southern Guinea Savannah (SGS), Ibadan in Derived Savannah (DS) and Ikene in Forest Region (FR) agroecological zones. A brief description of these agroecological zones is provided elsewhere (Atehnkeng et al., 2008a). Field plots in Zaria and Mokwa were planted on 19 July, 2007 and 2008, while field plots in Ibadan and Ikene were planted on 12 September in 2007 and 2008. The maize genotype, ACCR-9931-SR, was planted in all experiments and within plots, rows were 0.75 m apart, while plants were 15–20 cm apart within rows. Fields were fertilized with a compound fertilizer at a rate of 60 kg/ha NPK and plants were top-dressed at about 4 weeks after planting with urea (40 kg N/ha). At each location, the treated plot measured 90 m × 84 m (~0.75 ha), while the control (untreated) plot measured 56 m × 45 m (~0.25 ha) (Fig. 1). At each location, different fields were used in 2008 and 2009 and treated and control plots were about 1 km apart. The separation distance was necessary to avoid interplot interference due to the potential of atoxigenic isolates to move from treated to control plots (Bock et al., 2004). The physical segregation of the treated and control plots results in an experimental design referred to as clump segregation (Hurlbert, 1984). At about 40–45 days after planting (~2 to 3 weeks before flowering), field plots were inoculated by broadcasting the experimental product containing the four atoxigenic isolates on soil surfaces beneath the crop canopy at the rate of 10 kg/ha per isolate, (i.e., 40 kg/ha of the end use product). Prior to field inoculation, field operations were completed including hand cultivation, fertilization, and earthing up. This ensured that the product containing the strain mixture would remain on the soil surface. At each location in both treated and control plots, nine subplots (5.25 m × 6.5 m) were marked (Fig. 1) from which soil samples from top 2.5 cm layer of top soil were collected from at least 15 points before inoculum application. Within the treated plot, the inoculum was applied to all the nine subplots and each subplot was considered as replication. Within the treated plot, the inoculum was applied to all the nine subplots, while the control plot was untreated. The experiment was laid out as a clumped segregation (Hurlbert, 1984) and each subplot was considered as a pseudoreplication (Hurlbert, 1984). At harvest, 25 maize cobs were randomly collected from the nine subplots for the analysis of the *Aspergillus* strain populations and aflatoxins in the harvested grains.

2.3. Mycoflora analysis in soil and maize grain

Soil samples (each about 300 g) were oven dried with forced air at 48–50 °C for 48 h and placed in a mortar where the soil was pulverized to remove clods and then homogenized by manual mixing in polyethylene bags. Grain samples (500 g per sample) were ground using a blender (Waring Commercial, Springfield, MO, USA) to less than 20 μm particles. Members of *Aspergillus* section *Flavi* in soil and grain samples were isolated on Modified Rose Bengal Agar (MRBA) using a dilution plate technique as described by Donner et al. (2009). Briefly, 1 g of ground sample was suspended in 10 ml of sterile distilled water in 40 ml glass vials and mixed thoroughly in a vortex mixer for about 20 min and appropriate dilutions plated on MRBA in 9 cm diameter Petri plates. Plated Petri plates were incubated in the dark at 31 °C for 3 days. Members of *Aspergillus* section *Flavi* were initially identified based on colony morphology and the number of colony-forming units (CFU) of each species recorded. Further characterization was conducted for plates with less than 10 colonies, by transferring all individual colonies in each plate onto 5/2 agar and incubating them in the dark at 31 °C. After 5 days, isolates were classified as *A. flavus* L- or S strain, S_{BG} strain, *A. parasiticus* or *Aspergillus tamarii* based on colony morphology, sclerotial and conidial characteristics (Cotty, 1989).

2.4. Monitoring and recovery of fungal strains

To determine which isolate collected from soil and grain samples above belonged to the VCGs of the applied atoxigenic strains, 32 nitrate non-utilizing (*nit*$^-$) mutants were generated for L strain

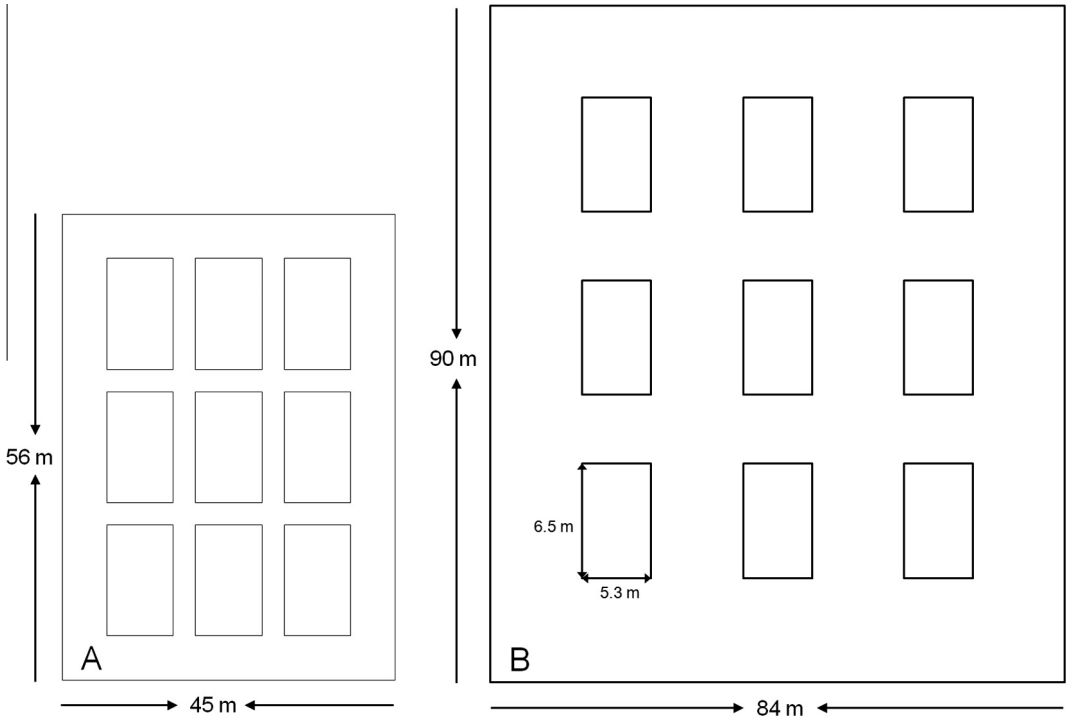

Fig. 1. Field layout of the experimental plots; (A) control plot where no atoxigenic strains of *Aspergillus flavus* were applied and (B) treated plot inoculated with a mixture of four atoxigenic strains of *A. flavus*. The entire area in the treated plot in each location was inoculated. Soil and maize grain samples were collected only in the 6.5 × 5.3 m^2 subplots. Control plots were located at least 1 km away from the treated plots and both plots were separated by cultivated and uncultivated fields.

isolates as described by Bayman and Cotty (1991) and Cotty (1994). Briefly, about 20 µl of each spore suspension was transferred to a well at the center of a Petri plate containing the selection medium (Czapek-dox broth, with 25 g/l $KClO_3$, 50 ml/l rose Bengal and 20 g/l Bacto Agar and pH adjusted to 7.0). Most isolates spontaneously sectored into nit mutant auxotrophs within 15 days at 31 °C and those that failed to produce mutants were re-inoculated at the center of another plate containing the selection medium. Auxotrophic sectors were transferred to the selection medium (Czapek-dox broth, with 15 g/l $KClO_3$, and 20 g/l Bacto Agar and pH adjusted to 6.5) for three days at 31 °C in order to clean up and stabilize the mutants. Mutants were subsequently grown on 5/2 agar for 3–5 days, and agar plugs stored in sterile water at room temperature until their use in complementation tests. Assignment of isolates to one of the VCGs of the applied atoxigenics was based on the complementation between *niaD*$^-$ (deficient in structural gene for nitrate reductase) and *cnx*$^-$ (deficient in a molybdenum cofactor) tester mutant of each applied VCG (Cove, 1976). In this study, a single complementation test was performed on a plate where three wells (3 mm in diameter), 2 cm apart, were cut in a triangular pattern in the center of the medium (20–25 ml) in 9 cm plastic Petri plates. Two wells were separately seeded with 20 µl tester pair (one tester per well) spore suspension of each of the four VCGs, while the third well was seeded with *nit* mutant of the isolate to be characterized. The complementation medium consisted of Czapek-dox broth adjusted to pH 6.0; solidified with 2% Bacto-agar (Difco) and supplemented with 1 ml of micronutrients. Complementation occurred within 10 days at 31 °C and *nit* mutant of an isolate complementing with one or both testers of an atoxigenic VCG belonged to the same VCG and was assumed to be the atoxigenic strain that was applied in the field. A total of 15,824 isolates were assessed for complementation in 2007 and 2008.

2.5. Ear rot assessment and aflatoxin analysis in harvested grain

Maize plots in Zaria and Mokwa were harvested on 13 September 2007 and 24 September 2008, while Ikene and Ibadan plots were harvested on 22 January 2008 and 6 January 2009. Harvesting was done manually by cutting the lowest internode of all the maize plants in the subplots, and vertically heaping them for further drying in the field according to farmers' practices. After one month, 25 ears were collected from each subplot in both the control and treated plots for recovery of applied atoxigenics strains as described above and for aflatoxin analysis. Aflatoxin analysis was conducted on two sets of grain: (i) well stored grain at harvest (in 2007 and 2008), and (ii) poorly stored grain (in 2008 only) where five cobs with husks were wetted for 4 h and allowed to dry slowly over a 10-day period, followed by oven drying at 48 °C for 48 h. After poor storage, the cobs were dehusked and rated for Aspergillus ear rot on a scale of 1–5 (Menkir et al., 2008) where 1 = least colonized and 5 = most colonized. Grain colonization by *Aspergillus* species was recorded by shelling five cobs and counting the number of visibly colonized grains in a sub-sample of 200 grains.

Aflatoxins in the two sets of grain were extracted as described by Atehnkeng et al. (2008b) where samples were ground using high speed blender (Waring Commercial, Springfield, MO, USA) and a 20 g sample blended with 70% methanol–water (70/30 vol/vol) for 3 min. The mixture was then passed through Whatman paper No. 1 and the filtrate collected in a 250-ml separation funnel. The solution was extracted with 25 ml methylene chloride and the methylene chloride partition was filtered through 40 g of anhydrous sodium sulfate to remove residual water. The extraction was performed twice and the extracts pooled in a polypropylene cup and evaporated to dryness in a fume hood in the dark. The residue was dissolved in 1 ml of methylene chloride and either diluted or concentrated to allow for accurate densitometry following separation of aflatoxins on thin layer chromatography plates. Aflatoxins were quantified using scanning densitometer, CAMAG TLC Scanner 3 with winCATS 1.4.2 software (Camag AG, Muttenz, Switzerland) and the minimum detection limit was 0.1 ng/g.

2.6. Data analysis

Soil population densities (CFUs), frequency of members of *A. flavus* section *Flavi*, recovery (%) of atoxigenic VCGs groups and aflatoxin concentrations (response variables, x) were transformed using the equation $y = \log_{10}(1 + x)$ to stabilize the variance prior to data analysis, while cob ratings for infection by *Aspergillus* and percentage of colonized grains were not transformed before analysis. To limit the type 1 error due to segregation of the treatment associated with the clump segregation design (Hurlbert, 1984) and the inherent differences between the locations in the four agroecological zones (Atehnkeng et al., 2008a), data were analyzed separately for each location. Differences in all response variables between treated and untreated control were tested by analysis of variance (ANOVA) using the PROC GLM procedure in SAS (version 9.1, SAS Institute Inc., Cary, NC, USA). Means of the response variables obtained from treated and control plots were separated using Student's t-test.

3. Results

3.1. Population densities in soil and grain

Natural soil densities (i.e., populations prior to inoculation) of *Aspergillus* section *Flavi* varied across agroecological zones (Table 1). In 2007, natural populations were higher in NGS and SGS than in FR and DS, while in 2008, the lowest densities were observed in SGS. Generally, population densities were lower in plots before inoculation and higher in inoculated plots at harvest. Prior to inoculation, population densities ranged from 31 to 7000 CFU/g in 2007 and from 144 to 1894 CFU/g in 2008. In 2007, there was no significant ($P > 0.05$) difference in soil population densities between field plots before inoculation in all locations except in Mokwa in SGS zone. In 2008, differences in natural soil populations between fields plots were significant ($P < 0.05$) in all locations except in Ibadan in DS zone (Table 1).

Population densities of *Aspergillus* section *Flavi* in harvested grains were higher in treated plots than untreated plots (Table 1). In 2007, differences in population densities in harvested grains between treated and untreated plots were only significant ($P < 0.05$) at Ikene field plots, while in 2008, these differences in densities in harvested grain between treated and untreated plots were significant ($P < 0.05$) in all locations except in Zaria in NGS zone.

3.2. Distribution of Aspergillus section Flavi

In both years, the most abundant members *Aspergillus* section *Flavi* in both soil and harvested grain were L- strains of *A. flavus*, while *A. parasiticus* was the least abundant (Table 2). Application of atoxigenic strains in the field also altered the quantity and composition of members of *Aspergillus* section *Flavi*. For example, in 2008, densities of L strain isolates in the soil increased from 62.7% prior to inoculation to 99.1% at harvest, while those of S_{BG}, *A. parasiticus* and *A. tamarii* decreased from 11.8% to 0.6%, 3.3% to 0.0%, and 22.0% to 0.2%, respectively. In both years, soil population densities of L strain isolates at harvest were also significantly ($P < 0.05$) higher in treated than control plots (Table 2), while soil densities of S_{BG}, *A. parasiticus* and *A. tamarii* were lower in treated than in control plots. A similar trend in population densities was

Table 1
Populations of *Aspergillus* section Flavi in soil before application of the atoxigenic strain biopesticide and in soil and maize grain at harvest in four agroecological zones in Nigeria.

Location	AEZ[a]	Treatment[b]	*Aspergillus* section Flavi colony-forming units (CFU) per gram[c]			
			2007		2008	
			Soil (prior to inoculation)	Grain (at harvest)	Soil (prior to inoculation)	Grain (at harvest)
Zaria	NGS	Control	656	1102	1764	739
		Treated	1567^{NS}	2337^{NS}	189^{*}	1867^{NS}
Mokwa	SGS	Control	7000	3160	475	6740
		Treated	917^{*}	16500^{NS}	144^{*}	40661^{*}
Ikene	FR	Control	31	37564	1894	6411
		Treated	35^{NS}	193644^{*}	177^{*}	180061^{*}
Ibadan	DS	Control	170	235177	1022	4833
		Treated	140^{NS}	315722^{NS}	1535^{NS}	164878^{**}

[a] AEZ = Agroecological zone; DS = Derive savannah, FR = Forest Region, NGS = Northern Guinea savannah, and SGS = Southern Guinea savannah.
[b] Control treatment refers to plot in which no atoxigenic strains were applied; treated refers to plots to which the biopesticide containing four atoxigenic strains was applied at the rate of 40 kg/ha.
[c] Colony forming units (CFU) were log transformed ($\log_{10}$[CFU + 1]) prior to analysis to stabilize the variance. Significance levels, $^{*}(P < 0.05)$ and $^{**}(P < 0.01)$ are for testing for differences between the populations of *Aspergillus* section *Flavi* in treated and control plots within a location based on Student's *t*-test; NS = not significant.

Table 2
Distribution of *Aspergillus* section *Flavi* strains in soil before application of the atoxigenic strain biopesticide and in soil and maize grain at harvest in 2007 and 2008 cropping seasons.

Strain[a]	Treatment[b]	2007[c]			2008[c]		
		Soil		Grain	Soil		Grain
		Before inoculation	At harvest	At harvest	Before inoculation	At harvest	At harvest
L	Control	63.1	46.3	87.8	50.5	48.6	95.9
	Treated	75.9^{*}	96.2^{*}	99.8^{*}	62.7^{*}	99.1^{*}	99.7^{NS}
S_{BG}	Control	11.2	19.7	12.0	12.5	18.8	2.5
	Treated	7.5^{NS}	2.1^{*}	0.1^{*}	11.8^{NS}	0.6^{*}	0.0^{NS}
A. parasiticus	Control	7.9	10.1	0.0	5.0	1.6	0.0
	Treated	1.2^{*}	0.1^{*}	0.0	3.3^{NS}	0.0^{*}	0.0
A. tamarii	Control	17.6	23.7	0.0	31.9	30.9	1.5
	Treated	15.2^{NS}		0.1^{NS}	22.0^{*}	0.2^{*}	0.2^{NS}

[a] L strain of *A. flavus* typically with smooth conidial surface and either an average sclerotial diameter >400 μm or without sclerotia; S_{BG} = Un-named taxon of *Aspergillus*.
[b] Control treatment refers to field plots in which no atoxigenic strains were applied; treated refers to plots to which the biopesticide containing four atoxigenic strains was applied at the rate of 40 kg/ha.
[c] Frequency of strains (%) in soils before inoculation, at harvest and grain samples in each plot. Soil and grains were processed and plated on modified rose Bengal agar. Thirty-two isolates were transferred to V-8 juice agar and identified morphologically after five days of incubation. Frequency numbers (x) were log transformed ($\log_{10}[x + 1]$) prior to analysis to stabilize the variance. Significance levels, $^{*}(P < 0.05)$, and $^{**}(P < 0.01)$ are for testing for differences between the populations of *Aspergillus* section *Flavi* in treated and control plots within a location based on Student's *t*-test; NS = not significant.

observed in harvested grains except that the absolute numbers for S_{BG}, *A. parasiticus* and *A. tamarii* in grains were much lower than those observed in the soil. In addition, population densities in harvested grains in treated and control plots were not significantly ($P > 0.05$) different in both years except for L- and S_{BG}-strains in 2007 (Table 2).

3.3. Recovery of applied atoxigenic strains from soil and grain

In both years, recovery of VCGs of applied atoxigenic strains from soil was very low across all locations (range 2.0–10.8%) prior to application of atoxigenic strains to field plots, except in 2008 at Ibadan where the recovery was 42% (Table 3). No significant ($P > 0.05$) differences in the recovery of VCGs of applied atoxigenics were observed between plots prior to application of strains except in Ibadan in 2008. Recovery in the soil and harvested grain increased dramatically after application of atoxigenic strains in all locations in both years. For example, recovery of VCGs of applied atoxigenics from the soil following application of atoxigenic strains ranged from 66.3% at Ibadan to 73.6% at Ikene in 2007. The corresponding recovery levels from control plots were significantly ($P < 0.05$) lower than those in treated plots and ranged from 0.0% at Ibadan to 5.6% at Zaria in 2007. A similar trend in the recovery of the VCGs of applied atoxigenics from soil was also observed in 2008 with recovery levels in treated plots ranging from 66.0% to 79.5% (Table 3). Similarly, recovery of VCGs of applied atoxigenics in harvested grain was significantly higher in treated plots (range 65.7–85.4%) than untreated plots (range 3.6–36.1%) across all locations in 2007 and 2008. Recovery rates of VCGs of applied atoxigenic strains from harvested grains in control plots were also much higher in 2008 (range 22.9–36.1%) than in 2007 (3.6–10.4%) (Table 3).

Prior to inoculation, recovery rates of VCGs of individual applied atoxigenic strains from the soil were consistently very low (range 0.3–8.0%) and not significantly different ($P > 0.05$) between treated and control plots in 2007 and 2008 (Table 4). However, recovery rates of respective VCGs increased after application of the biocontrol formulation in both years. Further, recovery rates of VCGs of each applied atoxigenic strains were significantly ($P < 0.05$) higher in treated than control plots throughout the study. In treated plots, VCGs belonging to La3279 and La3304 were the two most recovered in the soil at harvest in 2007 with rates of 17.1% and 23.1%, respectively, while in 2008, VCGs belonging to La 3279 and Ka16127 were the two most recovered with rates of 26.8% and

Table 3
Recovery (%) of atoxigenic VCGs in soil before application of the atoxigenic strain biopesticide and in soil and maize grain at harvest in four locations in Nigeria.

Location	AEZ[a]	Treatment[b]	2007[c]			2008[c]		
			Soil		Grain	Soil		Grain
			Before inoculation	At harvest	At harvest	Before inoculation	At harvest	At harvest
Zaria	DS	Control	6.5	5.6	3.9	4.5	31.6	23.7
		Treated	3.3^{NS}	70.6**	79.2**	5.2^{NS}	66.0*	65.7*
Mokwa	FR	Control	2.2	1.7	3.6	2.1	1.7	22.9
		Treated	3.6^{NS}	72.2**	79.2**	10.8^{NS}	75.4**	85.4*
Ikene	SGS	Control	3.6	0.7	10.4	6.3	32.7	36.1
		Treated	4.2^{NS}	73.6**	78.5**	3.1^{NS}	68.4*	67.7*
Ibadan	NGS	Control	2.0	0.0	10.3	3.7	11.9	25.5
		Treated	10.1^{NS}	66.3**	83.3**	42.0*	79.5**	78.1*
Mean[d]		Control	3.6	2.0	7.1	4.2	19.4	27.1
		Treated	5.3	70.7	80.1	15.3	72.3	74.2

[a] AEZ = agroecological zone; DS = Derive savannah, FR = Forest Region, NGS = Northern Guinea savannah, and SGS = Southern Guinea savannah.
[b] Control treatment refers to plot in which no atoxigenic strains were applied; treated refers to plots to which the biopesticide containing four atoxigenic strains was applied at the rate of 40 kg/ha.
[c] Recovery data (y) were log transformed ($\log_{10}[y+1]$) prior to analysis to stabilize the variance. Significance levels, $^{*}(P<0.05)$, and $^{**}(P<0.01)$ are for testing for differences between the populations of *Aspergillus* section *Flavi* in treated and control plots within a location based on Student's t-test; NS = not significant.
[d] Mean recovery of atoxigenic strains from the control and treated plots across all locations and AEZ.

Table 4
Recovery (%) of atoxigenic vegetative compatible groups of *Aspergillus flavus* in soil before application of the atoxigenic strain biopesticide and in soil and maize grain at harvest in four locations in Nigeria.

VCG[a]	Treatment[b]	2007			2008		
		Soil[c]		Grain[c]	Soil[c]		Grain[c]
		Before inoculation	At harvest	At harvest	Before inoculation	At harvest	At harvest
La3279	Control	0.8	0.3	3.2	0.9	12.1	11.8
	Treated	2.7^{NS}	17.1*	32.1*	8.0^{NS}	26.8^{NS}	25.6*
La3303	Control	0.7	0.3	0.3	0.3	2.9	2.0
	Treated	0.6^{NS}	14.1*	4.3*	2.2^{NS}	9.0*	10.5*
La3304	Control	0.7	0.7	1.4	1.7	3.4	7.0
	Treated	1.0^{NS}	23.1*	24.6*	1.5^{NS}	17.7*	16.7*
Ka16127	Control	1.3	0.7	2.1	1.2	1.0	6.1
	Treated	1.1^{NS}	16.3*	19.1*	3.6^{NS}	18.8*	21.3*
Total[d]	Control	3.5	2.0	7.0	4.1	19.4	26.9
	Treated	5.4	70.6	80.1	15.3	72.3	74.1

[a] VCG = vegetative compatible group.
[b] Control treatment refers to plot in which no atoxigenic strains were applied; treated refers to plots to which the biopesticide containing four atoxigenic strains was applied at the rate of 40 kg/ha.
[c] Recovery data (y) were log transformed ($\log_{10}[y+1]$) prior to analysis to stabilize the variance. Significance levels, $^{*}(P<0.05)$, and $^{**}(P<0.01)$ are for testing for differences between the populations of *Aspergillus* section *Flavi* in treated and control plots within a location based on Student's t-test; NS = not significant.
[d] Total recovery of all VCGs in the control and treated plots in soil before and at harvest and from maize grains at harvest.

18.8%, respectively (Table 4). Recovery rates of all VCG groups in the grain (mean 77.1%) at harvest were slightly higher than those from soil (71.4%). In both years, the most recovered VCG in grain was that of La3279 with rates of 32.1% and 25.6% in 2007 and 2008, respectively. The VCG belonging to La3303 was the least recovered in soil and grain in both years. Recovery rates of individual VCGs of the atoxigenic strains in soil or grains at harvest from control plots were low (range 0.3–12.1%).

3.4. Ear infection and aflatoxin contamination in grain

No significant ($P>0.05$) differences between treated and control plots were observed in the severity of *Aspergillus* ear rot on harvested ear and the number of colonized grains at all locations (Table 5).

Concentrations of aflatoxins were much higher in very poorly stored grains than in well stored grain at all locations (Table 6). Further, grain harvested in Zaria and Mokwa had highest levels of contamination, while grain harvested at Ikene had the lowest level of contamination in either treated (4.7 ng/g) or control (53.8 ng/g) plots. Across all locations, contamination of grains was significantly ($P<0.05$) lower in treated plots (range 11.6–343.2 ng/g) than in control plots (range 27.1–7560.9 ng/g) irrespective of storage conditions. The highest reduction of aflatoxins following application of atoxigenic strains was observed in Mokwa in 2007 where contamination was reduced by 99.2%, while the lowest reduction in contamination was observed in Ikene where contamination was reduced by 57.2% (Table 6). Reduction in contamination was generally much higher in very poorly stored grains (range 93.5–95.6%) than in well stored grains (range 57.2–99.2%) at all locations.

4. Discussion

Four atoxigenic strains of *A. flavus* were applied under field conditions in four agroecological zones in Nigeria to determine the efficacy of a mixed formulation in mitigating aflatoxin contamination of maize. This is the first study to demonstrate that mixtures of atoxigenic strains of *A. flavus* in a product can be used to reduce aflatoxin contamination in maize under field conditions. All four

Table 5
Rating of poorly stored maize cobs for Aspergillus ear rot and colonization of grains by Aspergillus species in control and treated plots in four agroecological zones in 2008 in Nigeria.

			Variable[a]	
Location	AEZ[b]	Treatment[c]	CobRating[d]	Colonized grains[e] (%)
Zaria	NGS	Control	3.6	63.9
		Treated	3.2^{NS}	50.7^{NS}
Mokwa	SGS	Control	3.3	40.7
		Treated	2.7^{NS}	43.6^{NS}
Ikene	FR	Control	4.1	46.2
		Treated	3.9^{NS}	47.8^{NS}
Ibadan	DS	Control	3.0	30.0
		Treated	3.3^{NS}	31.5^{NS}

[a] Significance test indicated by $^{*}(P < 0.05)$ is for differences between the response variable in treated and control plots within a location based on Student's *t*-test; NS = not significant.
[b] AEZ = agroecological zone; DS = Derive savannah, FR = Forest Region, NGS = Northern Guinea savannah, and SGS = Southern Guinea savannah.
[c] Control treatment refers to plot in which atoxigenic strains were not applied; treated refers to plots to which the biopesticide containing four atoxigenic strains was applied at the rate of 40 kg/ha.
[d] Based on a scale of 1 to 5 in which 1 = least colonized and 5 = most colonized. Presented rating value is a mean of five cobs.
[e] Based on a sample of 200 grains and values shown are a mean of nine counts.

atoxigenic strains were successful in competitively excluding aflatoxin producing strains from the grain although one strain was less efficient. The proportion of the *A. flavus* population composed of the L strain isolates increased, while the highly toxic S_{BG}-strain isolates and *A. parasiticus* decreased as a result of applications. A larger proportion of isolates belonging to VCGs of the four applied atoxigenic strains was recovered from soil in treated than control plots. There was also a corresponding reduction in aflatoxin contamination in harvested grain in treated versus control plots indicating a significant displacement of toxigenic strains that were able to infect the crop. Reduction in aflatoxin contamination was very high and ranged from 57% to 99% across the growing seasons. This study is also the first field evaluation of the ability of native atoxigenic strains to serve as an aflatoxin management tool in their region of origin in Africa. Endemic atoxigenic strains eliminate potential environmental risks usually associated with the use of exotic strains in biological control of plant diseases.

Maize kernels come into contact with *A. flavus* prior to harvest and the fungus remains on grains throughout harvest and in storage (Lillehoj, 1987). Thus, the potential for aflatoxin contamination exists before and after harvest. In this study, the recovery of atoxigenic VCGs of the applied atoxigenic strains from the grain at harvest was very high (>70%). Our results indicate that the significant reduction in aflatoxin contamination in grain in this study was due to the efficacy of the mixture of the atoxigenic strains in replacing toxigenic strains in the soil and grains thereby reducing aflatoxin producing potential of the *Aspergillus* community.

In Africa, poor grain handling practices during post-harvest stages is an important factor contributing to high and frequent levels of contamination. For example, predominance of fungi with the S strain morphology in the Eastern province of Kenya coupled with harvesting of maize with high moisture content and delayed drying of grains were among the important factors that contributed to extremely high levels of aflatoxin in 2004 when 124 people died of aflatoxicoses (Probst et al., 2011). Similarly, untimely rain during the postharvest periods was one of the major reasons for declaring 2.3 million bags of bags untradeable due high levels of aflatoxin contamination in 2010 in Kenya (Manyong et al., 2012). Significantly, the efficacy of the atoxigenic strain mixture in reducing aflatoxin contamination was pronounced (>93%) in treatments where dried maize grains were exposed to a simulated breakdown in storage conditions. When grains become wet during any of the postharvest grain handling stages, the *Aspergillus* population on and in grains multiply thus increasing aflatoxin production. Grains harvested from fields treated with a mixture of atoxigenic strains contain a high proportion of applied atoxigenic strains that grow when conditions are conducive but do not produce aflatoxins. These observations indicate the potential use atoxigenic strains of *A. flavus* to reduce postharvest infection and subsequent contamination by toxigenic strains resulting in high reduction in aflatoxin accumulation in stored grain. As such, postharvest application of atoxigenic strains of *A. flavus* may have the potential to prevent postharvest contamination of maize by strains associated with the crop in the field.

A. flavus was the most common member of *Aspergillus* section *Flavi* isolated from the soil and maize grains in untreated fields. Our results are similar to those reported in peanut where *A. flavus* was the predominant crop colonizer of peanuts even in soils treated with atoxigenic strains of *A. parasiticus* (Dorner and Horn, 2007). Results from the present study and from the study by Dorner and Horn (2007) along with previous inoculation studies (Calvert et al., 1978) all suggest that *A. flavus* is more aggressive than *A. parasiticus*. In a previous study in Nigeria (Atehnkeng et al., 2008a), the *A. flavus* L strain was also found to constitute >90% of

Table 6
Concentrations (ng/g) of B-aflatoxin ($B_1 + B_2$) in stored maize grains harvested from control plots and plots treated with the biopesticide containing a mixture of four atoxigenic strains of *Aspergillus flavus* in four locations in Nigeria in 2007 and 2008.

Location	AEZ [c]	Treatment [d]	2007[a]		2008a[a]		2008b[b]	
			B-aflatoxin [e]	Reduction (%)	B-aflatoxin [e]	Reduction (%)	B-aflatoxin[e]	Reduction (%)
Zaria	NGS	Control	992.4		72.5		7560.9	
		Treated	147.2**	85.2	10.7*	85.3	343.2**	95.5
Mokwa	SGS	Control	2792.4		50.2		2481.2	
		Treated	23.4**	99.2	6.9*	86.2	148.9**	94.0
Ikene	FR	Control	27.1		53.8		956.1	
		Treated	11.6**	57.2	4.7*	91.3	62.2***	93.5
Ibadan	DS	Control	95.2		41.7		2408.3	
		Treated	29.0**	69.5	11.4*	72.6	104.7**	95.6

[a] Aflatoxin analysis was conducted in grains at harvest.
[b] Following 3 months of storage after harvest, grains were wetted for 4 h and allowed to dry slowly either over a 10-day period.
[c] AEZ = agroecological zone; DS = Derive savannah, FR = Forest Region, NGS = Northern Guinea savannah, and SGS = Southern Guinea savannah.
[d] Control treatment refers to plot in which no atoxigenic strains were applied; treated refers to plots to which the biopesticide was applied at the rate of 40 kg/ha.
[e] Mean aflatoxin concentration in maize grains from nine sub-samples per plot. Aflatoxin reduction (%) = [1 − (aflatoxin in treated plot)/aflatoxin in control plot] × 100. Significance test indicated by $^{*}(P < 0.05)$ and $^{**}(P < 0.01)$ are for differences between the aflatoxin concentrations in treated and control plots based on Student's *t*-test.

the *Aspergillus* section *Flavi*. Thus, it is likely that most of the aflatoxin contamination of maize observed in this study was due to toxigenic L strain isolates of *A. flavus*. Similar conclusions were reported in a study to determine the efficacy of atoxigenic strains of *A. flavus* and *A. parasiticus* on aflatoxin contamination where *A. flavus* was determined to be responsible for most of contamination that occurred in peanut (Dorner and Horn, 2007). It is also important to note that up to 20% of population densities in soil and grain at harvest in untreated plots was of the unnamed taxon S_{BG}. Fungal strains belonging to the West African strain S_{BG} are phylogenetically divergent from but morphologically similar to other fungi with S strain morphology (Probst et al., 2011). The S_{BG} -strain produces large quantities of both B- and G-aflatoxins (Cotty and Cardwell, 1999). Thus, given the fairly moderate proportion of S_{BG} isolates observed in the current study, S_{BG} is likely an important causal agent of aflatoxin contamination observed in Nigerian maize.

Densities of *Aspergillus* section *Flavi* propagules in control plots were not related to the levels of aflatoxin contamination in the grain. For example, the section *Flavi* densities in grain from control plots in Zaria and Mokwa in 2007 were between 1100 and 3100 CFU/g with contamination ranging from 992 to 2792 ng/g, while the Ikene populations averaged 37,564 CFU/g with a corresponding contamination of only 27.1 ng/g. Differences in proportions of toxigenic and atoxigenic strains between Ikene, Mokwa and Zaria could explain the lack of proportionality between population densities and aflatoxin production. It is likely that the proportion of toxigenic compared to atoxigenic strains was higher at Zaria and Mokwa than in Ikene and soil populations in treated plots were not necessarily dominated by atoxigenic strains. These differences in the proportion of toxigenic and atoxigenic strains could also explain partly, for example, why non-significant differences in grain population densities of members of *Aspergillus* section *Flavi* in treated and untreated plots in Ibadan in 2007 resulted in a significant reduction in aflatoxin contamination. In cottonseed, maize and peanuts, population densities of toxigenic *A. flavus* and *A. parasiticus* have been found to be linearly related to aflatoxin concentration (Cotty, 1994; Dorner and Horn, 2007; Probst et al., 2011). However, the proportion of toxigenic and atoxigenic strains vary widely across agroecological zones in Nigeria (Atehnkeng et al., 2008a) and thus, it would have been interesting to determine the levels of toxigenic and atoxigenic strains in treated and control plots and relate these to toxin production in grain.

The success of biocontrol of aflatoxin in agricultural systems using atoxigenic strains relies on the ability of atoxigenic strains to displace toxigenic strains either in the soil or in the grain. Displacement of toxigenic strains by biocontrol strain can be achieved if the biocontrol strain has equal or better aggressive colonization traits that toxigenic strains possess naturally (Cotty, 2006; Cotty and Bayman, 1993). In this study, the recovery of the four atoxigenic VCGs from soil and harvested grain in treated plots was high (~75%). This indicates that the biocontrol strains used in this study have a competitive advantage over resident populations (toxigenic or atoxigenic) within *Aspergillus* section *Flavi* and they can provide long-term reduction in aflatoxin contamination. The biocontrol strains used in this study, La3279, La3303, La3304 and Ka16127 are indigenous strains isolated in Nigeria. Compared to exotic strains, biocontrol strains of indigenous VCGs are usually adapted to local cropping systems and can successfully compete for local resources (Mehl et al., 2012; Probst et al., 2011). This allows for a broader and more consistent displacement of atoxigenic strains by indigenous than exotic strains of *A. flavus*. Classical selection of biocontrol agents to control plant diseases has emphasized the use of single strains of the biocontrol agent (Ojiambo and Scherm, 2006). However, due to the variability in environmental niches, biocontrol is expected to be more effective when mixtures rather than single atoxigenic strains are used in disease control. There are several reasons that can account for the greater biocontrol activity of a mixture of strains compared to individual strains. Closely related microorganisms are able to coexist due to divergent adaptations to different ecological niches or life strategies (Johnson et al., 2006; Miller et al., 2009). In *A. flavus*, VCGs vary in their ability to: (i) colonize and disperse and (ii) deeply invade host tissues and the best colonizers are not the best sporulators (Mehl and Cotty, 2010). Furthermore, VCGs differ in adaptation to different cropping systems (Mehl and Cotty, 2013). As such, greater diversity of introduced VCGs will result in a diverse and potentially stable community able to compete and which is resilient to changes that occur during or between the growing seasons. Thus, modified *Aspergillus* communities with multiple atoxigenic VCGs are expected to have greater stability than those formed with single VCGs (Probst et al., 2011; Mehl et al., 2012). The greater stability and resilience of multiple atoxigenic VCGs over years will eventually provide long-term reductions in aflatoxin contamination. This is particularly important for small-scale growers in developing countries who may not have the necessary resources to periodically apply biocontrol products to their fields.

In the present study, we observed that a mixture of atoxigenic strains of *A. flavus* have a competitive advantage and are more aggressive than toxigenic strains in maize colonization. A similar observation has also been reported in peanut (Dorner and Horn, 2007). Of the four atoxigenic strains used in this study, La3303 was the least recovered in the soil or grain. Thus, future product formulations of the atoxigenic strain mixture should not include La3303 since it is an inefficient competitor. Strain mixtures involving only La3279, La3304 and Ka16127 and if necessary another competitive atoxigenic strain should still result in high reduction in aflatoxin contamination in the grain. Further, the high densities of *Aspergillus* section *Flavi* propagules in the treated plot was primarily due to the high application rate (40 kg/ha) for the formulated product. This application rate is two to four times higher than the recommended rates for other aflatoxin biocontrol products such as AF36. We recommend that the constituent isolates in the mixture are blended in equal proportion to generate an application rate of 10 kg/ha for the formulated product. It is expected that periodic application of atoxigenic strains will be required to maintain a high atoxigenic to toxigenic strain ratio to achieve long-term reduction in aflatoxin contamination. However, no studies have been conducted to determine the frequency of application over different cropping seasons that will maintain a high ratio of atoxigenic to toxigenic strains that leads to sustainable reductions in aflatoxin contamination. Further, it has been suggested that competitive exclusion of aflatoxin producers by atoxigenic strains during an increase in epidemic of *Aspergillus* ear rot allows atoxigenic strains to compensate for uneven application and to have influences beyond fields treated with atoxigenic strains (Cotty and Antilla, 2003). More studies are also needed to determine the extent to which this biocontrol benefit can influence the composition of fungal communities and particularly the soil and grain densities of aflatoxin producers in surrounding untreated maize fields.

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