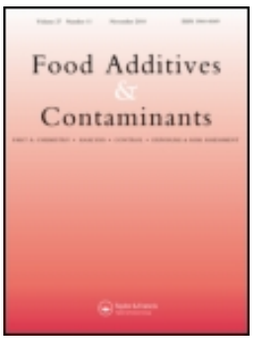

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Evaluation of atoxigenic isolates of *Aspergillus flavus* as potential biocontrol agents for aflatoxin in maize

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Aflatoxin contamination resulting from maize infection by *Aspergillus flavus* is both an economic and a public health concern. Therefore, strategies for controlling aflatoxin contamination in maize are being investigated. The abilities of eleven naturally occurring atoxigenic isolates in Nigeria to reduce aflatoxin contamination in maize were evaluated in grain competition experiments and in field studies during the 2005 and 2006 growing seasons. Treatments consisted of inoculation of either grains in vials or ears at mid-silking stage in field plots, with the toxigenic isolate (La3228) or atoxigenic isolate alone and co-inoculation of each atoxigenic isolate and La3328. Aflatoxin $B_1 + B_2$ concentrations were significantly ($p < 0.05$) lower in the co-inoculation treatments compared with the treatment in which the aflatoxin-producing isolate La3228 was inoculated alone. Relative levels of aflatoxin $B_1 + B_2$ reduction ranged from 70.1% to 99.9%. Among the atoxigenics, two isolates from Lafia, La3279 and La3303, were most effective at reducing aflatoxin $B_1 + B_2$ concentrations in both laboratory and field trials. These two isolates have potential value as agents for the biocontrol of aflatoxin contamination in maize. Because these isolates are endemic to West Africa, they are both more likely than introduced isolates to be well adapted to West African environments and to meet regulatory concerns over their use throughout that region.

Keywords: aflatoxin; corn; mycotoxin; competitive exclusion; West Africa

Introduction

Aflatoxin contamination of maize (*Zea mays* L.) results from growth of aflatoxin-producing isolates of the fungus *Aspergillus flavus* in maize kernels (Diener et al. 1987). This fungus is commonly found in soil and crop debris, which acts as the principal source of primary inoculum for infecting maize (Jaime-Garcia and Cotty 2004; Horn 2007). Isolates of *A. flavus* vary greatly in aflatoxin production, with some producing copious amounts and others none (Bayman and Cotty 1993; Dorner 2004; Barros et al. 2006; Horn 2007; Atehnkeng et al. 2008). Aflatoxin contamination can occur during crop development when the crop is either damaged (e.g., by insects) or stressed by heat and drought and after maturation when the crop is exposed to high moisture and high temperature either before harvest or in storage (Payne 1992). In addition to being potent hepatotoxic and carcinogenic metabolites, aflatoxins also impact on child growth and development and adversely affect immune status of people (Wild 2007).

Aflatoxin contamination of maize grain destined for human consumption and animal feed is heavily monitored and regulated in many countries to ensure a safe supply of food and feed (Food and Agriculture Organization (FAO) 2004). In Africa, regulatory controls are largely ineffective (Strosnider et al. 2006). Concerns over food safety and economic losses associated with aflatoxin contamination have led to development of strategies to control aflatoxin in crops (Robens and Riley 2002; Robens and Brown 2004; Cotty et al. 2008) including maize (Hell et al. 2008). Atoxigenic isolates (defined as isolates that do not produce any aflatoxin) of *A. flavus* have been developed as biological control agents directed at competing with and displacing aflatoxin producers. In an earlier study, reduction in aflatoxin production was demonstrated in corn inoculated with atoxigenic isolates before inoculation with toxigenic isolates under *in vitro* conditions (Brown et al. 1991). The potential for biological control of aflatoxin has been demonstrated under field conditions in cotton (Cotty 1994), peanut

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(Pitt and Hocking 2006; Dorner and Horn 2007) and corn (Brown et al. 1991; Dorner and Cole 1999; Abbas et al. 2006). Because competitive exclusion and multiple-year influences are components of biocontrol with atoxigenic isolates, locally adapted atoxigenics are thought to be necessary for optimal efficacy and long-term modifications to fungal community structures. Furthermore, the use of native atoxigenic isolates also alleviates some of the concerns about safety and environmental impacts that might be of greater concern for introduced organisms. Systematic studies on the use of native atoxigenic isolates of *A. flavus* in West Africa to reduce aflatoxin contamination of maize are needed in order to bring this aflatoxin management with atoxigenic isolates closer to practical use in that region (Bandyopadhyay and Cardwell 2003). In a previous study on the distribution and toxigenicity of *Aspergillus* section *Flavi* from maize kernels in Nigeria (Atehnkeng et al. 2008), the present authors identified many atoxigenic isolates of *A. flavus* that could be evaluated as potential biological control agents for the reduction of aflatoxin contamination in maize. The current study sought to evaluate the potential of several of the atoxigenic *A. flavus* isolates from Nigeria to reduce aflatoxin contamination of maize.

Materials and methods

Fungal isolates

The atoxigenic isolates of *A. flavus* (eleven in total) used in this study were isolated from maize kernels collected from farmers' stores in the Derived Savannah (six isolates), Southern Guinea Savannah (four isolates), and Northern Guinea Savannah (one isolate) agro-ecological zones of Nigeria (Table 1) during a previous study (Atehnkeng et al. 2008). All atoxigenic isolates belonged to the L strain of *A. flavus* (Cotty 1989). A lack of aflatoxin production was confirmed on single-spore transfers of each atoxigenic isolate in A&M medium containing 22.5 mM urea as the sole nitrogen source as previously described (Cotty and Cardwell 1999; Probst et al. 2007; Atehnkeng et al. 2008). A minimum of two fermentation experiments – one in stationary culture (5 ml fermentation medium in 20 ml vial) and the second in shake culture (70 ml medium in 250 ml Erlenmeyer flasks shaken at 150 rpm, 31°C) – were carried out. Briefly, dry extracts and aflatoxin standards were separated on thin-layer chromatography (TLC) plates (silica gel 60, 250 μm), by development with diethyl ether–methanol–water (96:3:1), visualized under ultraviolet light, and scored visually for the presence or absence of aflatoxin. The limit of detection for the 5 ml fermentations was 5 ng g^{-1} and for the 70 ml fermentations it was 0.4 ng g^{-1}. The L strain isolate La3228 from Derived Savannah was included as the toxigenic control (Table 1).

Stability of atoxigenicity in atoxigenic isolates

Three single-spore colonies from each of the eleven atoxigenic isolates were established on 5/2 agar medium (5% V8 juice and 2% agar, pH 5.2). Single spores from each of the 33 cultures were transferred on to 5/2 medium, cultured for 1 week (31°C) and again transferred by single spore. Similar serial transfers were repeated 20 times for each of 33 atoxigenic lines. Cultures from every fifth generation (i.e., fifth, tenth, 15th and 20th) were tested for aflatoxin production in 70-ml fermentations as described above.

Inoculum preparation

Spores from 5-day-old cultures (5/2 agar, 31°C) were suspended in sterile distilled water containing 0.02% Tween 80. The concentration of resulting spore suspension was adjusted to 10^6 spores ml^{-1} using a haemocytometer.

Table 1. Origins of isolates of *Aspergillus flavus* utilized in the current study.

Isolate	Toxigenicity	District in Nigeria	Latitude	Longitude	Agroecological zone*
Ab2216	Atoxigenic	Abuja	9°32′ N	7°25′ E	SGS
Ak2757	Atoxigenic	Akwanga	9°04′ N	8°38′ E	SGS
Ak3020	Atoxigenic	Akwanga	8°75′ N	8°55′ E	SGS
Ak3058	Atoxigenic	Akwanga	8°75′ N	8°55′ E	SGS
Ka16127	Atoxigenic	Kaduna	11°36′ N	8°56′ E	NGS
La3108	Atoxigenic	Lafia	8°65′ N	5°56′ E	DS
La3279	Atoxigenic	Lafia	8°46′ N	8°54′ E	DS
La3303	Atoxigenic	Lafia	8°49′ N	8°55′ E	DS
La3304	Atoxigenic	Lafia	8°49′ N	8°55′ E	DS
Lo4216	Atoxigenic	Lokoja	7°91′ N	6°76′ E	DS
Og0222	Atoxigenic	Ogbomosho	8°07′ N	4°18′ E	DS
La3228	Toxigenic	Lafia	8°46′ N	8°54′ E	DS

Note: *SGS, NGS and DS refer to Southern Guinea, Northern Guinea and Derived Savannah agroecological zones, respectively.

Competition on grains in the laboratory

Efficacy of atoxigenic isolate was initially assessed on harvested grain of maize genotype ACR 97 TZL *Comp1* under laboratory conditions. Grain was obtained from the maize breeding unit of the International Institute of Tropical Agriculture (IITA), Ibadan. Grain moisture was adjusted to about 25% by soaking the grains in water for 2 h using a modification of the method described by Abbas et al. (2006). For each replicate of each treatment, 30 maize grains (about 8 g) were autoclaved (121°C, 60 min) in a 120 ml Pyrex glass vial. The sterilized grains were seeded with aliquots of spore suspensions of toxigenic and atoxigenic isolates. There were 24 treatments including a water control with 0.02% Tween 80, twelve treatments for each isolate seeded individually (200 µl, 1×10^6 spores ml^{-1}), and eleven treatments for each atoxigenic isolate seeded with an equal quantity of the aflatoxin-producing isolate La3228 (100 µl each, 2×10^6 spores ml^{-1}). In the co-inoculated treatments, the toxigenic isolate was inoculated 1 h before the atoxigenic isolates. After seeding, vials were shaken in a Vortex mixer for 30 s to ensure kernel surfaces were completely coated with inoculum. Vial caps were loosened to ensure gas exchange. Vials were incubated (31°C for 7 days), oven dried (45°C, 1 day) to halt fungal activity, and prepared for aflatoxin analysis. Treatments were arranged in a completely randomized design with five replications and the experiment was conducted twice.

Competition on cobs in the field

Experiments were carried out on a ferric luvisol soil at the IITA research farm at Ibadan, which lies in the Derived Savannah zone, 7°30′ N latitude and 3°54′ E longitude (Moormann et al. 1975). Field plots were manually sown with maize genotype ACR 97 TZL *Comp1* (two seeds per hill) in single-row plots, 3 m long on 13 December 2005 and 28 September 2006. Plots were spaced 0.75 m between rows and 0.25 m between hills. Plants were thinned to one per hill 2 weeks after sowing and a compound fertilizer was applied to the plots at a rate sufficient to provide 60 kg ha^{-1} each of N, P, and K. An additional 30 kg ha^{-1} N (urea) was applied as top dressing 4 weeks after planting. At mid-silking stage, each developing ear was wounded in the centre once by pushing a cork borer (3 mm diameter) through the cob husk to a depth of 5 mm. Each ear was inoculated by applying 10 µl of spore suspension containing 1×10^6 conidia ml^{-1} to the wound with a pipetter. The 24 treatments were identical to those for the grain competition trial in the laboratory. The co-inoculation treatment was inoculated with 10 µl of inoculum containing 1×10^6 conidia ml^{-1} each of both the atoxigenic and toxigenic isolates. All treatments were arranged in a randomized complete block design with ten replications.

Plots were irrigated with overhead sprinklers every 3–4 days until 110 days after planting. Subsequently, irrigation was stopped 25 days before crop harvest to apply drought stress for predisposing plants to aflatoxin contamination. At maturity on 19 April 2006 (Test 1) and 26 February 2007 (Test 2), five cobs per plot were harvested and sun-dried in the husk for 14 days. Two sets of grains were evaluated to determine the ability of atoxigenic isolates to competitively exclude the aflatoxin-producer and thus prevent aflatoxin contamination both at the wound site and in unwounded kernels into which aflatoxin-producers spread. The first set of kernels (hereafter referred to as 'inner ring') consisted of grains directly adjacent to and surrounding the point of inoculation (Figure 1). The second set of grains (hereafter referred to as 'outer ring') consisted of kernels directly adjacent to the inner ring. Kernels from the inner and outer rings were removed with the aid of spatula, put into separate envelopes (grains from five cobs in each treatment plot were pooled separately for inner and outer rings), and stored at 4°C until analysis.

Aflatoxin analysis

Before aflatoxin analysis, grains were washed in sterile distilled water under the laminar flow hood to remove

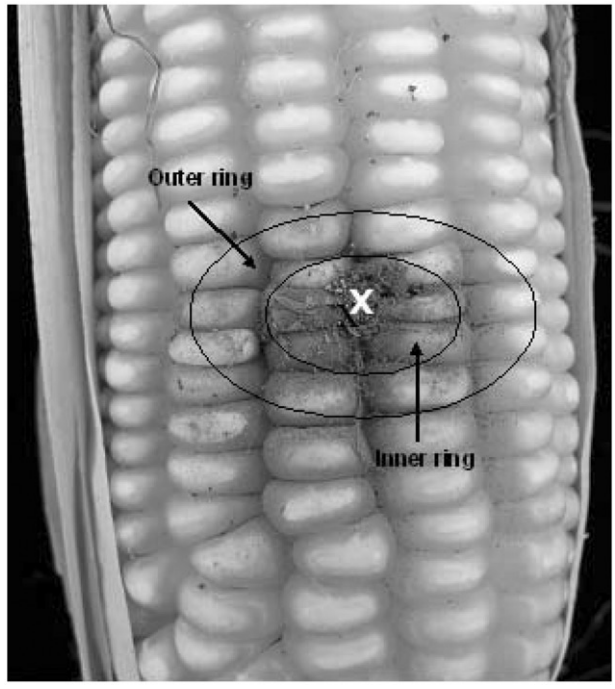

Figure 1. Maize cob inoculated with *Aspergillus flavus* at mid-silking stage at point '×' around which grains were sampled at maturity. Grains sampled in the inner ring were adjacent to and surrounding the point of inoculation, while grains in the outer ring were adjacent to the inner ring.

any spores and other surface contaminants that would be removed through normal grain processing in Nigeria. The cleaned grains were then oven dried at 45°C for 24 h. Aflatoxin analyses were performed using a method described earlier (Atehnkeng et al. 2008). Depending on the weight of the kernels, a 7–25-g sample was ground and extracted with a corresponding volume of 70% methanol (ratio of 1:5) using a high-speed blender (Waring Commercial, Springfield, MO, USA) for 3 min. The mixture was then passed through Whatman paper No. 1, the filtrate collected in a 250-ml separatory funnel, and 100 ml of distilled water were added to ease separation. The solution was extracted with 25 ml methylene chloride; the methylene chloride partition was filtered through 40 g of anhydrous sodium sulphate to remove residual water. The extraction was performed twice and the extracts were pooled in a polypropylene cup and evaporated to dryness in the dark, in a fume hood. The residue was dissolved in 1 ml of methylene chloride and either diluted or concentrated to allow accurate densitometry. Aflatoxin standards and extracts were separated on TLC plates as described above. Aflatoxins ($B_1 + B_2$) were quantified using scanning densitometer, CAMAG TLC Scanner 3 with winCATS 1.4.2 software (Camag AG, Muttenz, Switzerland), Aflatoxins G_1 and G_2 were not found in any samples. (Hereafter in the text, aflatoxin refers to aflatoxin B.) The minimum detection limit was 1.0 ng g^{-1}. Based on spiked recovery controls using 5, 10, 15, 20, and 25 ng g^{-1} levels, >85% of the aflatoxin present was recovered by this method.

Statistical analysis

Effects of treatments and test (grain competition) or years (field competition) on aflatoxin contamination were subjected analysis of variance using the PROC GLM procedure in SAS (version 9.1, SAS Institute Inc., Cary, NC, USA). Data from individual tests or years were analysed and presented separately if a significant treatment × test (or year) interaction was detected for aflatoxin contamination. Treatment means were separated using Fisher's protected least significant difference (LSD) test for inoculation treatments with atoxigenic isolates and water control as one group and co-inoculation treatments and inoculation with the toxigenic isolate as another group.

Results

Stability of atoxigenicity

None of the cultures obtained through 20 serial transfers of the eleven atoxigenic isolates tested positive for aflatoxin. This showed that the atoxigenic isolates used in the study were stable for the atoxigenicity trait.

Aflatoxin content in grain competition

Co-inoculation of atoxigenic isolates with the toxigenic isolate La3228 significantly ($p < 0.0001$) affected aflatoxin contamination in grain. There was also a significant ($p < 0.0001$) treatment × experiment interaction on aflatoxin contamination in maize grains and, thus, the results are presented separately for each experiment (Table 2). In the first experiment, grains co-inoculated with isolate La3279 and La3228 had 33 ng g^{-1} aflatoxin and this level of contamination was significantly ($p < 0.05$) lower than for all the remaining co-inoculation treatments. Grains inoculated with Ak2757 and La3228 had the most contamination (7205 ng g^{-1}) among the co-inoculation treatments but still significantly less than maize inoculated with the toxigenic isolate La3228 alone (24,068 ng g^{-1}). Aflatoxin reduction ranged from 70.1% for isolate Ak2757 to 99.9% for La3279. A similar trend in aflatoxin contamination was observed in the second experiment except that, overall, lower levels of contamination were observed in the second experiment. In addition, percent toxin reduction in the second experiment was higher than in the first experiment ranging from 96.4% for isolate Ak2757 to 99.8% for La3279 (Table 2). In both experiments, no aflatoxin contamination was detected in grains inoculated with any of the atoxigenic isolates alone further confirming inability of these isolates to produce aflatoxin in maize (data not shown).

Aflatoxin content in grains from field inoculated cob

There was no significant ($p = 0.87$) treatment × year interaction in aflatoxin contamination in grain inoculated either with single *A. flavus* isolate or in treatments where atoxigenic isolates were co-inoculated with the toxigenic isolate La3228. Results are, therefore, presented based on combined data over both years Grains from inoculated zones treated with atoxigenic isolates alone had very low levels of aflatoxin (range = 0.1–28.9 ng g^{-1}) and were not significantly different from water control treatment (Table 3). Higher aflatoxin concentrations occurred in the inner ring of grains compared with the outer ring of grains.

Co-inoculation with atoxigenic isolates significantly ($p < 0.0001$) reduced the quantity of aflatoxins in the grain at harvest compared with grain from ears inoculated with the aflatoxin-producing isolate La3228 alone (Table 3). Compared with the toxigenic La3228 that resulted in 5280 ng g^{-1} of aflatoxin, levels of contamination in grains in which the toxigenic isolate was co-inoculated with La3279 and La3303 were 37.6 and 40.6 ng g^{-1}, respectively (Table 3). Aflatoxin levels were significantly ($p < 0.05$) higher in the inner ring of grains compared with grains in the outer ring. The relative reduction in aflatoxin in maize grains across

Table 2. Aflatoxin B ($B_1 + B_2$) content and reduction in contamination in maize kernels co-inoculated with an aflatoxin producer and an atoxigenic isolate of *Aspergillus flavus* in glass vials under laboratory conditions.

	Experiment I		Experiment II	
Isolate[a]	Aflatoxin B ($ng\,g^{-1}$)	Aflatoxin reduction (%)[b]	Aflatoxin B ($ng\,g^{-1}$)	Aflatoxin reduction (%)
La3279	33.0	99.9	17.2	99.8
Og0222	793.4	96.7	110.0	98.7
Lo4216	830.9	96.5	147.4	98.3
Ab2216	1170.9	95.1	303.5	96.5
Ak3020	1241.8	94.8	294.4	96.6
La3303	1733.7	92.8	26.0	99.7
La3108	1762.4	92.7	153.1	98.2
Ka16127	1773.2	92.6	246.9	97.1
Ak3058	2232.6	90.7	109.4	98.7
La3304	2717.4	88.7	44.1	99.5
Ak2757	7205.1	70.1	307.0	96.4
La3228	24068.3	–[c]	8566.9	–
LSD ($\alpha = 0.05$)	548.4	–	462.6	–

Notes: [a] *Aspergillus flavus* La3228 is the aflatoxin producer and its corresponding aflatoxin content is based on grain inoculation using this single isolate.
[b] Aflatoxin reduction (%) = [1 − (total aflatoxin in co-inoculation/total aflatoxin in La3228)] × 100.
[c] No information presented since the parameter is not relevant.

Table 3. Aflatoxin B ($B_1 + B_2$) in maize either inoculated with an aflatoxin producer alone or co-inoculated with both an aflatoxin producer and an atoxigenic isolate of *Aspergillus flavus* under field conditions[a].

	Aflatoxin B ($ng\,g^{-1}$) in single inoculations[c]			Aflatoxin B ($ng\,g^{-1}$) in co-inoculations			
Isolate[a]	Inner grain ring	Outer grain ring	Total aflatoxin	Inner grain ring	Outer grain ring	Total aflatoxin	Reduction[d] (%)
La3279	0.3	0.2	0.5	37.0	0.6	37.6	99.3
La3303	26.3	2.6	28.9	39.1	1.5	40.6	99.2
Ab2216	11.1	5.6	16.7	156.1	77.4	233.5	95.6
0g0222	17.4	0.0	17.4	120.0	11.6	131.6	97.5
Lo4216	0.1	0.0	0.1	156.9	3.0	159.9	97.0
Ak3020	1.2	0.0	1.2	235.3	27.5	262.8	95.0
La3108	0.0	0.1	0.1	182.2	1.1	183.3	96.5
La3304	0.6	0.0	0.6	161.8	4.3	166.1	96.9
Ka16127	3.9	0.0	3.9	255.9	17.5	273.4	94.8
Ak3058	0.7	0.0	0.7	267.8	54.5	322.3	93.9
Ak2757	2.7	0.0	2.7	309.1	42.0	351.1	93.4
None	23.0	2.5	25.5	–	–	–	–
La3228	–[e]	–	–	4988.9	290.9	5279.8	n.a.
LSD ($\alpha = 0.05$)	26.9	5.3	32.2	538.3	80.7	619.0	–

Notes: [a] There was no significant isolate × year interaction and results shown are based on combined 2005 and 2006 data.
[b] *Aspergillus flavus* La3228 is the aflatoxin producer and its corresponding aflatoxin content is based on inoculation using this single isolate.
[c] Inner grain ring refers to grains directly adjacent and surrounding the point of inoculation, while outer grain ring refers to grains directly adjacent to the inner grain ring. Total aflatoxin is the sum of aflatoxin in grains in the inner and outer rings. Each value is the mean of two experiments, each with ten replications.
[d] Aflatoxin reduction (%) = [1 − (total aflatoxin in co-inoculation/total aflatoxin in La3228)] × 100.
[e] No information presented since the parameter is not relevant.

years was lowest and highest for the atoxigenic isolates AK2757 and La3279 with 93.4% and 99.3% reduction in contamination, respectively.

Discussion

Biological control of aflatoxin contamination with atoxigenic isolates of *A. flavus* has been practised for over a decade in commercial agriculture in several regions of the USA (Dorner 2004; Cotty 2006; Cotty et al. 2008). Atoxigenic isolate-based biological control seeks competitively to exclude aflatoxin producers from the crop environment to achieve both single-season influences on the aflatoxin content of the crop and long-term reductions in the average aflatoxin-producing potential of fungal communities resident in

target areas. Long-term influences permit cumulative benefits from applications across multiple years and can provide additional benefits by changing the fungal community to which both untreated rotation crops and nearby residents are exposed. In order to achieve such benefits, atoxigenic isolates must be adapted to both target crops rotations and the target environments. The current study provides the first attempt to identify systematically locally adapted endemic atoxigenic isolates of potential value in aflatoxin mitigation in West Africa where cropping systems include maize.

Specific highly efficacious isolates were identified in the current study. All the atoxigenic isolates evaluated in this study competed successfully with the aflatoxin producer and, as a result, substantially reduced aflatoxin concentrations both in harvested grain under controlled conditions and in developing maize kernels under conditions that favour high levels of aflatoxin contamination during crop production in Nigeria. The excellent efficacy observed for all the atoxigenic isolates examined in the current study combined with the large number of atoxigenic *A. flavus* isolates observed in prior studies (Donner et al. 2006; Atehnkeng et al. 2008) suggests that there is considerable genetic diversity from which to select isolates of value in Africa. These results indicate that biocontrol of *A. flavus* using atoxigenic isolates can be an effective aflatoxin management option in West Africa. Similar controlled environment and field studies have been reported previously for North American atoxigenic *A. flavus* isolates on maize (Brown et al. 1991), peanut (Dorner 2004; Dorner and Horn 2007), cotton (Cotty 1990, 1994), and in Australian isolates on peanut (Pitt and Hocking 2006).

La3279 was the most effective atoxigenic isolate from Nigeria in reducing aflatoxin contamination both in laboratory tests and during the two-year field study with an average aflatoxin reduction of >99.3%. Other atoxigenic isolates that had similar efficacy (>92%) in aflatoxin reduction were La3303, Og0222 and Lo4216. Although grains from ears co-inoculated with toxigenic and atoxigenic isolates had more aflatoxins than ears inoculated with atoxigenic isolates alone, the results also indicate that the atoxigenic isolates La3279, La3303 and La3108 are able to reduce the spread of the toxigenic isolate and resultant aflatoxin contamination from the point of inoculation to other parts of the maize ear as reflected in reductions in the aflatoxin content of outer ring kernels. The reductions in aflatoxin contamination observed in this study (70.0–99.9%) are similar in magnitude to reductions observed in other studies examining either direct inoculation of maize grains or ears. In an earlier study by Brown et al. (1991), an atoxigenic isolate reduced aflatoxin by 80–95% in co-inoculated ears compared with ears inoculated with an aflatoxin producer alone. In a recent similar study, Abbas et al. (2006) reported reductions of 65–95%. In peanut, application of atoxigenic *A. flavus* to soil reduced aflatoxin in kernels to about 93% of the toxigenic control (Dorner and Horn 2007). Although results from the later three studies can be compared with aflatoxin reduction observed in the present study, differences in atoxigenic and toxigenic isolates, their population densities and the environmental conditions in respective studies affected the magnitude of the reduction in aflatoxin contamination. Drought and high temperatures after silking generally enhance the potential for aflatoxin contamination in maize (Payne 1992), with considerable variation from year to year. Mean temperature and relative humidity during crop production were similar during the two years of the current study (28.3 and 26.3°C; and 74.6 and 68.3% for 2005 and 2006, respectively). These conditions are generally accepted as being within the range conducive for aflatoxin contamination (Hassan and Lloyd 1995).

The aflatoxin producer La3228 spread from the point of inoculation through the inner ring of kernels into the outer ring of kernels where it contaminated kernels with significant aflatoxin concentrations. Although most of the atoxigenic isolates were highly effective at reducing contamination of both inner ring and outer ring kernels, three isolates were appreciably less effective at reducing contamination of the outer ring kernels. The fourth most efficacious isolate at reducing contamination of inner ring kernels (average reduction = 98%), Ab2216, was the worse isolate at reducing contamination of the outer ring (average reduction = 73%). This may indicate a reduced ability to compete during ramification of host tissues and may provide an initial indication of less competitive ability during host colonization and, thus, less efficacy in practice. Comparisons of inner and outer ring efficacies may provide a rapid method for weeding out lesser competitive isolates before expensive and time-consuming field-release studies.

Concentrations of aflatoxins in kernels inoculated with the toxigenic strain ranged from 8500 to 24,000 ppb in the current study. These extreme aflatoxin levels were achieved because only infected kernels were analysed. In naturally infected maize a relatively low percentage of kernels become infected and contaminated by *A. flavus* and kernels are frequently infected by mixtures of high and low aflatoxin-producing isolates (Cotty 1994). Both these phenomena contribute to the lower aflatoxin concentrations found in naturally infected maize. Data from the current study clearly demonstrate how mixed infections can result in lower aflatoxin contamination levels. Even with the very high aflatoxin background incited in the current work, most of the examined atoxigenic isolates from West Africa achieved over 90% reductions in contamination. Achieving such reductions in maize routinely consumed in

West Africa would result in dramatically reduced exposure of the consuming populations to aflatoxins. When combined with other management practices such as resistant cultivars and proper storage, biocontrol may ultimately allow reduction of aflatoxin concentrations in maize grown in West Africa to below levels mandated by law in Europe and North America.

The competitiveness of the applied isolate in the process of colonizing and becoming established in crops is a vital aspect in the success of the use of atoxigenic isolates for biocontrol (Cotty and Bayman 1993; Dorner 2004). Reduction in aflatoxin occurs as a result of intra-specific competitive exclusion, i.e., displacement of the toxigenic isolates by the applied atoxigenic isolates as has been demonstrated for aflatoxin biocontrol through soil application of the atoxigenic isolate AF36 in cottonseed fields (Cotty 1994). Further research is required to establish whether the atoxigenics described in the present work prevent contamination during crop development by displacing the toxigenic isolates.

One of the theoretical considerations in the choice of atoxigenic isolates is the stability of the atoxigenic phenotype (Pitt and Hocking 2006). All of the eleven atoxigenic isolates examined in the current work were stable through 20 serial transfers on agar medium. Aflatoxin production is regulated by several genes and it is likely that these isolates have one or more defective genes (Ehrlich and Cotty 2004; Chang et al. 2005). Ideally, the atoxigenic isolates for safe use in biological control should belong to vegetative compatibility groups (VCG) that do not have toxigenic members (Cotty 2006). This precaution is to ensure that atoxigenic and toxigenic isolates within a VCG do not exchange genetic material and generate progenies that produce aflatoxins (Ehrlich et al. 2007). Further work is in progress in our laboratory to determine the toxigenicity of isolates that are members of the VCGs in which these eleven atoxigenic isolates belong. Low aflatoxin concentrations were detected in kernels inoculated in the field with the atoxigenic isolates alone and the water control. This contamination may be due to chance introduction of aflatoxin producers from the environment. This has also been observed in previous studies (Brown et al. 1991). Atoxigenic isolates may be applied either in a conidial formulation directly to the crop or, more commonly, on a nutrient source (i.e., barley or wheat seed) to the soil beneath the crop (Bock and Cotty 1999; Dorner 2004; Cotty 2006). In the present study atoxigenic isolates were introduced directly into wounds in maize ears along with an aflatoxin producer. Further research is needed to establish the extent of variability among these atoxigenic isolates in their ability to colonize developing maize crops after soil application. Studies are currently underway to evaluate a variety of locally available substrates as nutrient source/carrier for atoxigenic isolates for field application in West Africa.

The present study demonstrated that there are many atoxigenic isolates of *A. flavus* endemic to Nigeria that when co-inoculated with an aflatoxin producer under both laboratory and field conditions of West Africa are effective at reducing aflatoxin contamination in maize. The examined atoxigenics were isolated from the major maize producing regions of Nigeria. These isolates have the potential to become management tools for the biocontrol of aflatoxin contamination of maize in West Africa.

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