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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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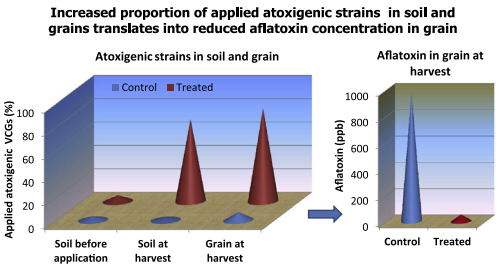
HIGHLIGHTS

- 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),