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The Atoxigenic Biocontrol Product Aflasafe SN01 Is a Valuable Tool to Mitigate Aflatoxin Contamination of Both Maize and Groundnut Cultivated in Senegal

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Abstract

Aflatoxin contamination of groundnut and maize infected by *Aspergillus* section *Flavi* fungi is common throughout Senegal. The use of biocontrol products containing atoxigenic *Aspergillus flavus* strains to reduce crop aflatoxin content has been successful in several regions, but no such products are available in Senegal. The biocontrol product Aflasafe SN01 was developed for use in Senegal. The four active ingredients of Aflasafe SN01 are atoxigenic *A. flavus* genotypes native to Senegal and distinct from active ingredients used in other biocontrol products. Efficacy tests on groundnut and maize in farmers' fields were carried out in Senegal during the course of 5 years. Active ingredients were monitored with vegetative compatibility analyses. Significant ($P < 0.05$) displacement of aflatoxin producers occurred in all years, districts, and crops. In addition, crops from Aflasafe SN01-treated fields contained significantly ($P < 0.05$) fewer aflatoxins both at harvest and after storage. Most crops from treated fields contained aflatoxin concentrations permissible in both local and international markets. Results suggest that Aflasafe SN01 is an effective tool for aflatoxin mitigation in groundnut and maize. Large-scale use of Aflasafe SN01 should provide health, trade, and economic benefits for Senegal.

Keywords: *Aspergillus flavus*, biological control, biopesticide, vegetative compatibility analysis

In warm agricultural areas, several economically important crops frequently become contaminated with aflatoxins produced by fungi belonging to *Aspergillus* section *Flavi* that also cause kernel rot. Aflatoxins are potent compounds that pose a myriad of serious health effects, including death, to both humans and animals (Bryden 2012; Wild 2002). Susceptible crops include maize, groundnut, chilies, cottonseed, and tree nuts (Bhatnagar et al. 1993; Cotty et al. 1994; Singh and Cotty 2019). Typically, susceptible crops become contaminated before harvest, and aflatoxin concentration continues to increase throughout storage if conditions are favorable for toxin formation (Bandyopadhyay et al. 2007; Diedhiou et al. 2011; Kachapulula et al. 2017a). Aflatoxin content of foods and feeds is monitored and regulated in most developed nations, but regulations in many nations in sub-Saharan Africa (SSA) are either nonexistent or poorly enforced because of consumption of self-grown crops in rural households; widespread presence of informal markets; and lack of appropriate infrastructure, qualified personnel, and economic incentive to quantify aflatoxins (Williams et al. 2004). When crops are contaminated with aflatoxin levels above maximum thresholds, farmers, traders, food processors, and consumers are negatively affected, because the crop products cannot be legally traded (Bandyopadhyay et al. 2016; Williams 2008).

The most common agent causing aflatoxin contamination is *Aspergillus flavus* (Amaike and Keller 2011). This species is subdivided into L and S morphotypes. The former produces copious conidia and a few large sclerotia (>400 μm), whereas the latter produces abundant small sclerotia (<400 μm) and scanty conidia (Cotty 1989). L-morphotype isolates vary in aflatoxin-producing potential, whereas S-morphotype isolates consistently produce high aflatoxin levels; both morphotypes produce only B aflatoxins (Cotty 1989; Probst et al. 2012). In SSA, species other than *A. flavus* play important roles in dictating aflatoxin contamination events. For example, a fungal lineage morphologically similar but phylogenetically divergent to the S-morphotype is native to West Africa. This lineage, called unnamed taxon S_{BG}, produces large concentrations of both B and G aflatoxins in maize, groundnut, and sesame seed (Cotty and Cardwell 1999; Diedhiou et al. 2011; Donner et al. 2009; Probst et al. 2012, 2014). The unnamed taxon S_{BG} was introduced as *Aspergillus aflatoxiformans* by Frisvad et al. (2019). *Aspergillus parasiticus* is also an important aflatoxin producer in maize and groundnut in some parts of Africa (Kachapulula et al. 2017b).

Aspergillus species (and morphotypes) are also subdivided into numerous vegetative compatibility groups (VCGs). Members of the same VCG descend from the same clonal lineage, and little variation among them exists (Grubisha and Cotty 2010, 2015; Leslie 1993). Certain L-morphotype VCGs are entirely composed of individuals that do not produce aflatoxins (atoxigenic) (Atehnkeng et al. 2016; Bandyopadhyay et al. 2016; Grubisha and Cotty 2015; Mehl et al. 2012). Diversity among VCGs in *Aspergillus* populations can be characterized using simple sequence repeat (SSR) markers. These markers are efficient and powerful in deciphering relatedness among strains and identifying genetic groups in a population. Strains can be differentiated into VCGs based on polymorphisms at SSR loci, because individuals belonging to an SSR haplotype or closely related haplotypes frequently belong to the same VCG (Grubisha and Cotty 2010, 2015).

Maize is among the primary staples, whereas groundnut is a major source of protein and a commonly grown cash crop in Senegal. Both crops frequently contain dangerous aflatoxin levels (Agbetiameh

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et al. 2018; Bandyopadhyay et al. 2007; Kachapulula et al. 2017b; Udomkun et al. 2017; Waliyar et al. 2015). Thus, large portions of children and adults in West African nations, including Senegal and neighboring The Gambia, are chronically exposed to high aflatoxin levels (Turner et al. 2000; Watson et al. 2015). Since the 1970s, groundnuts produced in Senegal rarely entered domestic and/or international premium markets because of high aflatoxin levels (Bandyopadhyay et al. 2016; Otsuki 2001; Xiong and Beghin 2012).

Reduced aflatoxin exposure and improved trade could be a reality if practical, efficient aflatoxin management strategies become available for use in groundnut and maize cultivated in Senegal. A strategy that uses native atoxigenic *A. flavus* L-morphotype isolates as biocontrol agents to competitively displace aflatoxin-producing isolates in the field allows production of crops with little to no aflatoxin content (Cotty 2006). The strategy has been adapted and improved for use in many nations in SSA (Bandyopadhyay et al. 2016). In the United States, biocontrol products are based on single atoxigenic L-morphotype genotypes (Cotty et al. 2007; Dorner 2004), whereas formulations used in SSA nations, under the trade name Aflasafe, contain four distinct atoxigenic L-morphotype genotypes (Bandyopadhyay et al. 2016). A multigenotype strategy is thought to have greater potential for long-term field establishment of *Aspergillus* communities with low aflatoxin-producing potentials (Mehl et al. 2012; Probst et al. 2011). The opportunity to develop a biocontrol product for Senegal arose when 1,000 isolates of *Aspergillus* section *Flavi* from two regions were characterized to determine the etiology of aflatoxin contamination in maize and sesame (Diedhiou et al. 2011). While quantifying the aflatoxin-producing potential of the 1,000 isolates, Diedhiou et al. (2011) detected 447 atoxigenic L-morphotype isolates, which served as the initial germplasm for the search for candidate biocontrol genotypes. The atoxigenic isolates in the germplasm were subjected to microbiological, physiological, and molecular analyses to identify atoxigenic genotypes with (i) wide distribution across Senegal, (ii) membership in diverse genetic groups (SSR/VCG), and (iii) superior ability to reduce aflatoxin contamination in maize and groundnut when coinoculated with an aflatoxin producer under laboratory conditions. Based on these studies, an isolate from each of the four superior African atoxigenic *Aspergillus* vegetative compatibility groups (AAVs) was selected as the active ingredient for the biocontrol product Aflasafe SN01.

A biocontrol product must be registered with the biopesticide regulatory authority before large-scale evaluation under typical farming practices. Key information required for regulatory approval includes methods to identify the constituent strains and efficacy of the product. In this study, we report the SSR signatures for identifying the VCGs of constituent strains of Aflasafe SN01 and the efficacy of Aflasafe SN01 in limiting aflatoxin concentrations in groundnut and maize cultivated in Senegal. Our experimental approach for product performance aimed to investigate whether applications of Aflasafe SN01 (i) efficiently limited aflatoxin contamination in both groundnut and maize cultivated in Senegal during production and throughout storage and (ii) increased frequencies of Aflasafe SN01 atoxigenic genotypes in treated fields. After approved for use, Aflasafe SN01 could serve as a valuable tool for reducing aflatoxin contamination of both groundnut and maize produced in Senegal.

Materials and Methods

Microsatellite genotyping. A total of 447 atoxigenic *A. flavus* L-morphotype isolates identified in a previous study (Diedhiou et al. 2011) were characterized using SSRs developed for *A. flavus* (Grubisha and Cotty 2009). DNA extraction, multiplex PCR, and microsatellite genotyping were conducted following previously described protocols (Callicott and Cotty 2015; Islam et al. 2018); >20% of isolates were subjected to at least three independent PCR and genotyping assays for all loci to assess consistency of the data. Allele frequencies and haplotypes were assessed with GenoDive (Meirmans and Van Tienderen 2004). Relationships among genotypes were displayed with a Neighbor-Net network generated with SplitsTree4 (Huson and Bryant 2006) based on chord distances calculated with GenoDive (Meirmans and Van Tienderen 2004).

Atoxigenic L-morphotype isolates. The population genetic analyses revealed 12 dominant atoxigenic SSR haplotypes widely distributed across Senegal. Representative strains of the SSR haplotypes were evaluated in their ability to reduce aflatoxin accumulation when challenged with highly toxigenic *A. flavus* isolates in kernel screening assays (KSAs) as described by Probst and Cotty (2012). In parallel, tester pairs of VCGs were developed for the SSR haplotype groups following previously described protocols (Bayman and Cotty 1991; Cove 1976). The four strains with superior ability to limit aflatoxin contamination when challenged with an aflatoxin producer were selected to be active ingredients of the biocontrol product Aflasafe SN01.

Genetic relationship among biocontrol isolates from the United States, Nigeria, and Senegal. A Cavalli–Sforza chord distance matrix obtained with Genodive was used to generate a Neighbor-Net network using SplitsTree 4.8 (Huson and Bryant 2006). Recombination and genetic distances among atoxigenic genotypes used in biocontrol formulations in the United States, Nigeria, and Senegal were evaluated with this approach, which uses a jackknife strategy and repeats the ϕ test after each individual is removed and subsequently replaced.

The biocontrol product and its manufacturing. Aflasafe SN01 was produced in the laboratory at the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria, as per the method described by Atehnkeng et al. (2014). Briefly, to prepare Aflasafe SN01, a batch of autoclaved sorghum grain was individually inoculated with a suspension containing spores of each of the four selected atoxigenic isolates, incubated at 31°C for 18 h, and dried in an oven at 55°C for 4 days. Equal proportions of dried grains separately inoculated with each isolate were mixed to constitute the product. The finished formulated product was placed in 2.5-kg polyethylene bags, sealed, and transported (air freight) to Senegal under appropriate export permit from the Nigeria Agricultural Quarantine Service and import permit from La Direction de Protection Végétaux (DPV) of Senegal.

The quality of the product (purity, sporulation, and composition of the active ingredient fungi) was determined as follows. Approximately 100 g of inoculated sorghum grains were collected per each 20 kg of finished product, and they were transferred to sterile plastic bags. Each sample was brought independently into a biological safety cabinet, and 100 sorghum grains were plated onto two plates each of 5-2 agar (5% V8 Juice [Campbell Soup Company] and 2% Bacto-agar [Difco Laboratories], pH 6.0), Nutrient Agar (Lam M; 28 and 20 g/liter glucose), and Violet Red Bile Agar (VRBA; Difco Laboratories; 41.5 g/liter, pH 7.4). The plates were incubated at 31°C for 7 days and examined to count the number of grains colonized by *A. flavus* and presence/absence of any other microorganism, including fecal coliforms on VRBA. Spore production was evaluated by placing 24 grains from each batch in individual wells in a 24-well cell culture plate and incubating as above. After incubation, three replicates of two seeds in the 24-well cell culture plates were rinsed three times with 10 ml of 100% ethanol. The resulting wash from each replicate was mixed with 10 ml of distilled water and poured into a turbidimeter vial. Spore yield was quantified by turbidity using an Orbeco-Helling digital direct reading turbidimeter (Orbeco Analytical Systems Inc.) and a nephelometric turbidity unit (NTU) versus colony-forming unit (CFU) standard curve ($y = 49{,}937x$; x = NTU; y = spores per milliliter).

From each subsample, 20 isolates were examined to assess membership in VCGs to which Aflasafe SN01 isolates belong. This was done using nitrate nonutilizing (*nit*) mutants, which were generated following previously described protocols (Atehnkeng et al. 2014, 2016). All recovered mutants were tested for membership in one of the four Aflasafe SN01 VCGs using vegetative compatibility assays. Fungal suspensions (15 µl containing ~150 spores) of each VCG tester pair and the mutant of interest were seeded into 3-mm-diameter wells 1 cm apart (in a triangular pattern) in starch agar (36 g/liter dextrose, 20 g/liter soluble starch, and 2% Bacto-agar, pH 6.0) (Cotty and Taylor 2003) and incubated for 7 days at 31°C. Mutants of isolates complementing a tester pair of a VCG were assigned to that VCG. Complementation was observed as a zone

of dense prototrophic growth where complementary mutants met and fused.

Field plots and Aflasafe SN01 application. Trials to examine the efficacy of Aflasafe SN01 in reducing aflatoxin in groundnut were conducted in Diourbel and Nioro districts in 2010 to 2013. In 2014, efficacy trials for aflatoxin control were conducted in groundnut in Tambacounda district and maize in Nioro district. Nioro and Diourbel are in the semiarid Sudan Savanna agroecological zone (AEZ), whereas Tambacounda is located in the Northern Guinea Savanna AEZ (Fig. 1). Crops produced in those regions are at risk of aflatoxin contamination (Diedhiou et al. 2011). Fields to be treated in any one year were carefully selected to ensure that the same field was not treated in the previous year so that potential carryover of Aflasafe SN01 isolates from one year to the next did not interfere with treatment effect.

Aflasafe SN01 was deployed in collaboration with members of farmers' associations. All farmers voluntarily consented to conduct Aflasafe SN01 efficacy trials. Farmers were advised to grow crops following their own agronomic practices without any special interventions. In general, every year farmers planted their preferred groundnut and maize varieties, which varied from region to region, after the onset of significant rainfall during mid-July. Farmers weeded the fields by hand or bullocks, top dressed with urea, and earthed up (i.e., piling up soil around the base of the plants) before application of Aflasafe SN01 to avoid burying the product.

The product was broadcasted by hand at a rate of 10 kg/ha 2 to 3 weeks before crop flowering, which occurred in all years during the second half of September. Farmers were trained to apply Aflasafe SN01 as described by Atehnkeng et al. (2014). For each treated field, a neighboring field >0.1 km apart was selected as the corresponding untreated control field; this avoided interference by biocontrol isolates moving from treated to control fields (Bock et al. 2004). The numbers of Aflasafe SN01-treated and control fields are given in Table 1. Field size ranged from 0.25 to 5 ha. In all years, crops were harvested during the first week of November. All fields were rainfall dependent.

Soil and crop sampling. Soil samples were collected before treatment to determine natural occurrence of VCGs to which Aflasafe SN01 active ingredients belong in the examined fields. This occurred in all years except 2013. Around 150 g of soil was collected by subsampling fields across transects from three random (40 to 50 subsamples) locations to a depth of 2 cm (Cotty 1997). Samples were air dried in the shade and then, sent to the laboratory in IITA Ibadan. After arrival, samples were dried in a forced air oven (2 days at 50°C) and transferred to a biological safety cabinet, where soil clods were eliminated with a hammer; then, samples were homogenized by hand within polyethylene plastic bags.

Crop samples were collected at harvest to determine influences of Aflasafe SN01 application on both fungal community structure and aflatoxin concentration of treated and control crops. Farmers harvested their crops and stacked them in the field for drying. Maize cobs and groundnut pods were stripped from plants in randomly selected stacks and shelled, and the grains were separated into two sets (~1 kg each). One set was immediately transferred to the DPV Plant

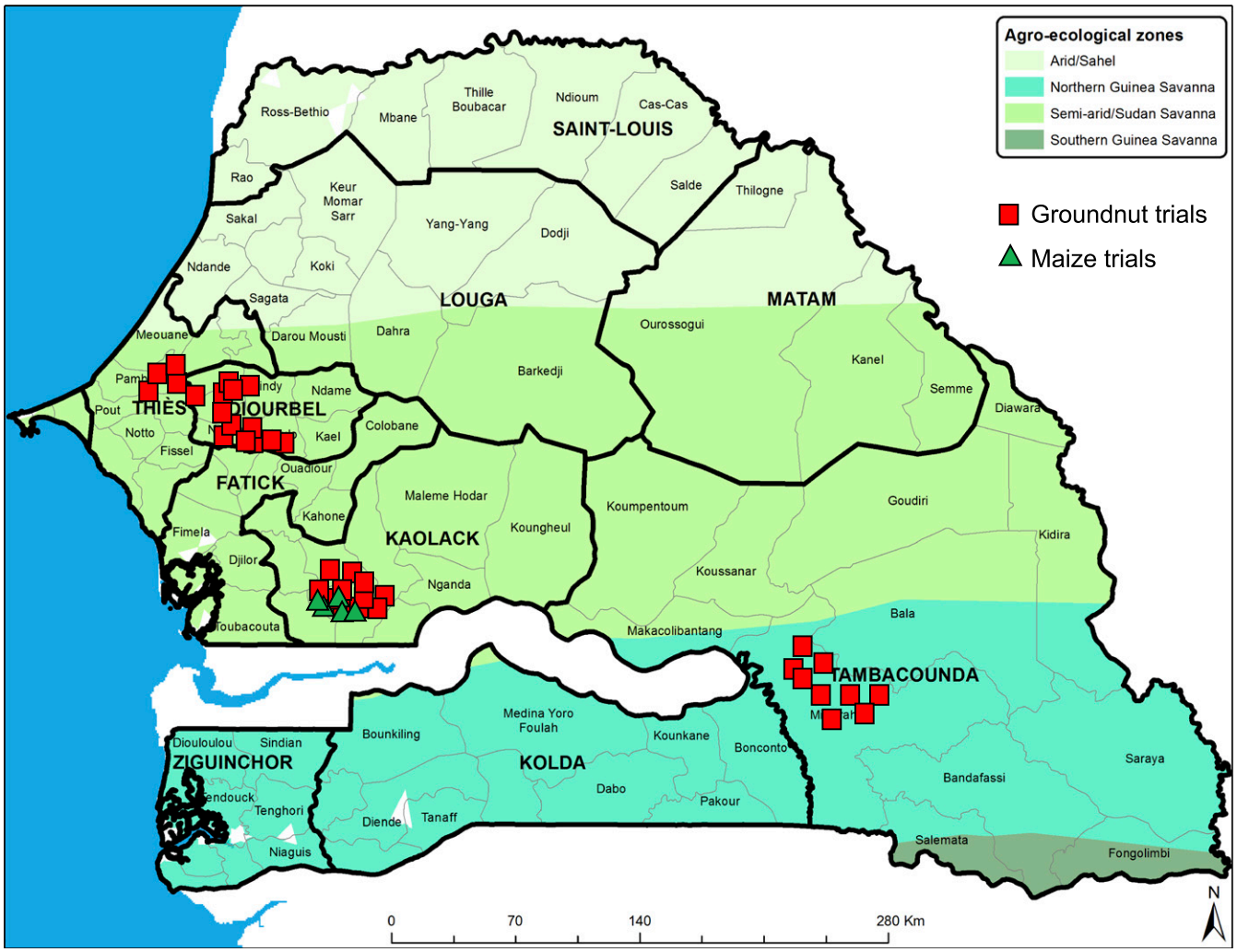

Fig. 1. Map of Senegal illustrating areas in which the biocontrol product Aflasafe SN01 was tested in groundnut and maize fields during 2010 to 2014. Symbols may represent multiple fields.

Pathology laboratory and kept at 4°C, whereas the other set was stored for 4 months in farmers' stores and collected after that period. Then, samples from both sets were ground (to pass through a 1-mm^2 sieve; Newark Wire Cloth Co.) using a laboratory blender (Waring Commercial) and sent to IITA Ibadan for aflatoxin and microbiological analyses.

Aflatoxin quantification. Aflatoxins were extracted from groundnut by combining 20 g of ground sample with 100 ml of 80% methanol (Dorner and Cole 1993), and they were extracted from maize by combining 20 g of ground sample with 100 ml of 70% methanol (Atehnkeng et al. 2008). Mixtures were agitated on a Roto-Shake Genie (Scientific Industries) for 30 min at 400 rpm. Then, mixtures were passed through fluted filter paper (Whatman paper No. 1). Aflatoxins were quantified as previously described using a scanning densitometer with accompanying software (TLC Scanner 3 with WinCATS 1.4.2 software; Camag) (Atehnkeng et al. 2008). Limit of detection of aflatoxin was 0.1 μg/kg.

Mean and variance of aflatoxin concentration of all samples within a treatment for a given crop and year were calculated. The percentages of samples containing aflatoxin levels of <4 μg/kg (European Union maximum level), <20 μg/kg (U.S. Food and Drug Administration action level), and >20 μg/kg (universally considered unacceptable for human consumption) were calculated for each treatment-crop-year combination. To calculate percentages, the numbers of samples with <4, 4 to <20, and >20 μg/kg were counted. The counts for each category were converted into percentage by multiplying the counts by 100 and dividing the product with the total number of samples in the specific treatment-crop-year combination.

Fungal examination. Fungi belonging to *Aspergillus* section *Flavi* were recovered from soil and grain samples by the dilution plate technique on modified rose Bengal agar (Cotty 1994a). Initially, 1 g of sample was suspended in 10 ml of sterile distilled water and vortexed for 30 s, and 100-μl aliquots were plated in triplicate. Adjustments to aliquot volume and/or sample quantity were made to obtain <10 *Aspergillus* section *Flavi* colonies per plate. Plates were incubated at 31°C for 3 days. For each sample, 16 discrete colonies were transferred onto 5-2 agar and incubated at 31°C for 7 days. Isolates were assigned to their corresponding species (*A. flavus* L-morphotype, *A. aflatoxiformans*, *A. parasiticus*, or *Aspergillus tamarii*) based on colony characteristics and spore ornamentation (Cotty 1989; Klich and Pitt 1988) as well as aflatoxin-producing potential using previously described protocols (Cotty and Cardwell 1999). Aflatoxin quantification was conducted as above. Incidences of *Aspergillus* section *Flavi* species in maize and groundnut samples were calculated as CFU per 1 g of sample. Isolates were saved as agar plugs (3 mm in diameter) of sporulating cultures in 4-ml vials containing 2 ml of sterile distilled water and maintained at room temperature.

Vegetative compatibility analyses. Frequencies of VCGs to which Aflasafe SN01 isolates belong were monitored using *nit* mutants, which were generated for all L-morphotype isolates recovered from both soil and grain samples as described above. All recovered mutants were tested for membership in each of the four Aflasafe SN01 VCGs as described above. Mutants of isolates complementing a tester pair of a VCG were assigned to that VCG.

Data analysis. Data on CFU, *Aspergillus* species distribution, incidence of Aflasafe SN01 VCGs, and aflatoxin concentration (response variable, x) were transformed using the equation:

$$y = \log_{10}(1 + x)$$

to stabilize the variance before statistical analysis. Means were separated using paired t tests (PROC T TEST, $\alpha = 0.05$) using SAS software (version 9.2, SAS Institute Inc.). Untransformed data are presented in summary tables and graphs in this paper. In all cases, comparisons were done between Aflasafe SN01-treated and control fields.

Results

Selection of atoxigenic strains composing Aflasafe SN01. The population genetic analyses of 447 atoxigenic *A. flavus* L-morphotype isolates identified previously by Diedhiou et al. (2011) revealed 12 atoxigenic groups widely distributed across Senegal. VCG grouping concurred with the grouping revealed by SSRs (data not shown). The four isolates that had the highest aflatoxin reduction when challenged with highly toxigenic *A. flavus* isolates in KSA were Ss19-14, MS14-19, M2-7, and M21-11. These isolates were selected to compose the biocontrol product Aflasafe SN01 and belong to VCGs AAV-SS19-14, AAV-MS14-19, AAV-M2-7, and AAV-M21-11, respectively. The four VCGs are native to six areas of Senegal: River Senegal Valley, Niayes, Bassin Arachidier, Ferlo, Senegal Oriental, and Casamance.

Genetic relationship among biocontrol strains. Allele calls from 17 loci distributed throughout the eight chromosomes of *A. flavus* (Grubisha and Cotty 2009) were compared among the Aflasafe SN01 isolates and biocontrol genotypes used for aflatoxin mitigation in the United States (*A. flavus* AF36 and NRRL21882; Afla-Guard) and Nigeria (Aflasafe) (Table 2). Each of the four Aflasafe SN01 isolates could be distinguished from other biocontrol isolates on the basis of allele calls for loci AF31, AF42, and AF64, because they were unique in the four Aflasafe SN01 isolates. Furthermore, SSR data were used to generate a Neighbor-Net network (Huson and Bryant 2006) that revealed genetic relationships among the 10 examined biocontrol isolates (Fig. 2). The network grouped Og0222 with NRRL 21882, and although La3279, La3304, Ka16127, and M2-7 were not tightly clustered, there was considerable distance between these isolates and the other six isolates.

Quality control of the product. All examined Aflasafe SN01 batches yielded 100% of carrier grains colonized by *A. flavus*. There were no other microorganisms recovered in any of the grains. The recovered *A. flavus* fungi were solely composed of the Aflasafe SN01 strains. Other genotypes of *A. flavus* were never detected. Each strain was found on 25 ± 3% carrier grains of the examined batches. Spore yield per gram of product was, on average, 3,500 ± 300 CFU.

Fungal densities in treated and control fields. Overall, *Aspergillus* population densities in soil before Aflasafe SN01 application were similar ($P > 0.05$) in treated and control fields except in Diourbel during 2011, where densities were higher in soils to be treated (Table 3). In general, in both treated and control field soils, fungal densities were always <9,000 CFU/g (range = 103 to 8,276).

In general, application of Aflasafe SN01 did not result in significantly higher ($P > 0.05$) fungal densities in treated crops compared

Table 1. Number of groundnut and maize fields treated with Aflasafe SN01 and accompanying control fields in three districts of Senegal[y]

	2010		2011		2012		2013		2014	
Crop and treatment	**Diourbel**	**Nioro**	**Diourbel**	**Nioro**	**Diourbel**	**Nioro**	**Diourbel**	**Nioro**	**Tambacounda**	**Nioro**
Groundnut										
Treated	18	18	20	20	17	21	18	42	50	–
Control	18	18	20	20	17	21	18	42	50	–
Maize										
Treated	–[z]	–	–	–	–	–	–	–	–	44
Control	–	–	–	–	–	–	–	–	–	44

[y] In all fields, soil samples were collected before treatment, except in 2013, and crop samples at harvest.
[z] Efficacy trials not conducted.

with control crops. Exceptions were noticed in Nioro in 2010 and 2011 as well as in Diourbel in 2011, although in the latter case, higher fungal densities were detected in soils before Aflasafe SN01 application. Visual inspection of grains from treated and control fields revealed no differences in fungal growth or moldiness when examined at harvest. In all cases, fungal densities were higher in grains at harvest than in soils before inoculation.

Distribution of *Aspergillus* section *Flavi*. Regardless of year and district, the *A. flavus* L-morphotype dominated all soils before inoculation (range = 96 to 100%) (Table 4). Other *Aspergillus* section *Flavi* fungi included *A. aflatoxiformans* (range = 0 to 3%), *A. parasiticus* (range = 0 to 0.1%), and *A. tamarii* (range = 0 to 2.5%). When comparing frequencies of each fungal type in treated and untreated soils, significant differences were not detected ($P > 0.05$) regardless of year and district (Table 4).

Examination of fungal types in grain at harvest revealed that the L-morphotype continued to dominate in treated (range = 77.5 to 100%) and control fields (range = 57.3 to 100%). In control fields, *A. aflatoxiformans* composed up to 42.7% of the population (Nioro 2012) (Table 4). Frequencies of *A. parasiticus* and *A. tamarii* were low (range = 0 to 4%) in the examined grains across districts and years regardless of treatment. *A. tamarii* was detected only in grains from treated fields (range = 0.1 to 4%). Frequencies of both L-morphotype and *A. aflatoxiformans* were significantly ($P < 0.05$) different between grains from treated and control fields only in Nioro (2011 and 2012) and Diourbel (2011).

Frequencies of Aflasafe SN01 strains in soil and grain. Natural frequencies of Aflasafe SN01 VCGs in soil before the initial application were low (range = 0 to 0.3%) (Table 5). In the rest of the years, combined frequencies of Aflasafe SN01 VCGs before Aflasafe SN01 application ranged from 2.5 to 37.2%. A significantly higher ($P < 0.05$) incidence of Aflasafe SN01 VCGs before application was detected in fields to be treated compared with the fields assigned as control in Diourbel 2012 and Tambacounda 2014 (Table 5). The combined recovery of Aflasafe SN01 VCGs was significantly ($P < 0.0001$) higher in all treated fields in comparison with control fields, regardless of region, crop, and year. Frequencies of Aflasafe SN01 VCGs in treated fields ranged from 42.2 to 72.9% (Table 5).

Aflatoxin contamination in grain at harvest and after storage. Aflatoxin concentrations were generally >20 μg/kg in the control fields, except in groundnut in 2013 in Diourbel and maize in 2014 in Nioro. In treated crops, the mean aflatoxin concentration in all but one case was <10 μg/kg in all years, districts, and crops (Table 6). Overall, significantly lower ($P < 0.05$ or less) aflatoxin concentrations occurred in crops from Aflasafe SN01-treated fields both at harvest and after storage (Table 6). At harvest, crops from treated fields contained 89% fewer aflatoxins than crops from control fields, and aflatoxin reductions ranged from 58.3% (Nioro 2010) to 100% (Nioro 2014). After storage, crops from treated fields contained 86.8% fewer aflatoxins than crops from control fields (range = 76.2 to 95.4%). Aflasafe SN01 was equally effective in reducing aflatoxins in all of the districts where it was tested. At harvest, the variance of aflatoxin concentration in crops from treated fields was 53.3 to 100% times lower than in the control fields. Furthermore, after storage, the variance of aflatoxin concentration in crops from treated fields was 93.4 to 99.9% times lower than in the control fields.

Crops from Aflasafe SN01-treated fields contained higher proportions of samples with <4 μg/kg aflatoxins both at harvest and after storage (Table 7). Indeed, at harvest, >75% of samples contained <4 μg/kg aflatoxins in comparison with 55.6% of samples from control fields across all locations and years. Even after storage, 73.9% of crops from treated fields contained <4 μg/kg aflatoxins. Overall, <6% of crops from treated fields contained >20 μg/kg aflatoxins compared with 24% crops from control fields both at harvest and

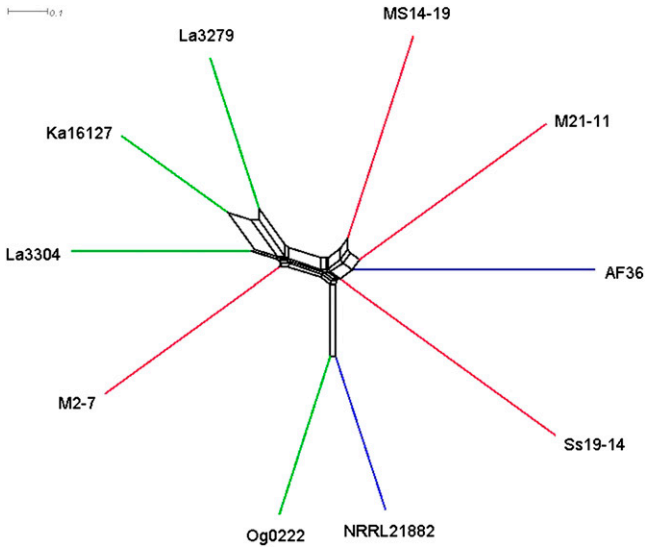

Fig. 2. Neighbor-Net network based on 17 simple sequence repeat loci showing relationships among 10 atoxigenic *Aspergillus flavus* genotypes used as active ingredients in biocontrol products. Network was generated with SplitzTree4 based on chord distances calculated with GenoDive. Genotypes with red lines are active ingredients of Aflasafe SN01. Green lines indicate active ingredients of Aflasafe. Blue lines indicate active ingredients of U.S.-registered products Afla-Guard (NRRL21882) and AF36 Prevail.

Table 2. Allele sizes of 17 simple sequence repeat loci (Grubisha and Cotty 2009) for active ingredients of biocontrol products AF36 Prevail, Afla-Guard, Aflasafe, and Aflasafe SN01[z]

Product	Active ingredients	AF28	AF13	AF43	AF22	AF31	AF53	AF34	AF42	AF8	AF16	AF54	AF17	AF11b	AF66	AF64	AF63	AF55
AF36 Prevail	AF36	119	161	385	188	308	134	310	162	177	191	168	353	163	269	213	135	174
Afla-Guard	NRRL21882	119	141	402	144	312	131	320	146	168	169	161	353	138	269	161	127	180
Aflasafe	Og0222	119	128	379	144	312	131	296	150	166	169	161	353	132	269	161	127	180
Aflasafe	La3279	135	145	385	192	346	134	301	181	189	169	161	356	141	261	169	129	180
Aflasafe	La3304	131	135	385	192	315	134	323	159	171	169	161	359	141	255	169	127	184
Aflasafe	Ka16127	135	145	385	192	367	134	301	159	160	169	161	362	141	261	169	127	184
Aflasafe SN01	M21-11	119	145	411	188	325	144	314	168	180	175	172	353	150	269	195	127	172
Aflasafe SN01	MS14-19	119	148	385	188	343	134	320	156	186	175	161	350	150	271	183	129	172
Aflasafe SN01	Ss19-14	135	148	387	208	349	154	310	223	151	206	176	353	138	271	209	127	176
Aflasafe SN01	M2-7	113	151	376	196	352	134	301	187	171	169	161	359	141	261	215	127	178

[z] AF36 Prevail and Afla-Guard are registered for use in the United States, Aflasafe is registered for use in Nigeria, and Aflasafe SN01 is registered for use in Senegal. Aflasafe and Aflasafe SN01 each contain four atoxigenic *Aspergillus flavus* genotypes.

Table 3. Densities of *Aspergillus* section *Flavi* in soil, groundnut, and maize collected from control and Aflasafe SN01-treated fields before biopesticide application and at harvest in three districts of Senegal where efficacy trials of Aflasafe SN01 were conducted from 2010 to 2014

	CFU/g[v]								
	2010		2011		2012		2013[w]	2014[x]	
District and treatment	Soil before inoculation	Grain at harvest	Soil before inoculation	Grain at harvest	Soil before inoculation	Grain at harvest	Grain at harvest	Soil before inoculation	Grain at harvest
Diourbel									
Treated	8,276 ns	119,035 ns	853*	156,332*	574 ns	21,823 ns	3,924 ns	–[y]	–
Control	2,120	11,576	431	22,385	959	26,717	3,703	–	–
Nioro									
Treated	576 ns	58,124*	434 ns	129,090*	1,054 ns	20,763 ns	2,772 ns	3,830 ns	228,175 ns
Control	103	6,908	326	11,325	476	9,398	2,272	4,811	7,767
Tambacounda[z]									
Treated	–	–	–	–	–	–	–	2,269 ns	26,889 ns
Control	–	–	–	–	–	–	–	2,298	32,102

[v] In each district, by individual year, CFU per 1 g from treated samples with an asterisk (*) significantly differed from its corresponding control treatment by Student's *t*-test ($\alpha = 0.05$). ns = non-significant.
[w] Soil samples were not collected before application of Aflasafe SN01 during 2013.
[x] Maize was collected only in Nioro district during 2014; groundnuts from this district were not sampled during 2014. The Diourbel region was not treated during 2014.
[y] Efficacy trials not conducted.
[z] The Tambacounda district was treated (groundnut) only during 2014.

Table 4. Frequencies of *Aspergillus* species distribution in soil, groundnut, and maize (Nioro 2014) samples collected from control and Aflasafe SN01-treated fields before biopesticide application and at harvest in three districts of Senegal from 2010 to 2014

	Aspergillus species/strain distribution (%)[x,y]							
	Soil before inoculation				Grain at harvest			
Year, district, and treatment	L	A	P	T	L	A	P	T
2010								
Diourbel								
Treated	99.7 ns	0.0 ns	0.0 ns	0.3 ns	100 ns	0.0 ns	0.0 ns	0.0 ns
Control	99.1	0.0	0.0	0.9	99.7	0.3	0.0	0.0
Nioro								
Treated	100 ns	0.0 ns	0.0 ns	0.0 ns	77.5 ns	18.5 ns	0.0 ns	4.0 ns
Control	100	0.0	0.0	0.0	76.2	23.8	0.0	0.0
2011								
Diourbel								
Treated	99.3 ns	0.0 ns	0.0 ns	0.7 ns	100*	0.0 ns	0.0 ns	0.0 ns
Control	97.5	0.0	0.0	2.5	96.7	1.8	1.5	0.0
Nioro								
Treated	97.5 ns	1.0 ns	0.0 ns	1.5 ns	99.2*	0.8*	0.0 ns	0.0 ns
Control	97.5	1.6	0.0	0.9	83	17.0	0.0	0.0
2012								
Diourbel								
Treated	98.4 ns	0.9 ns	0.0 ns	0.7 ns	100 ns	0.0 ns	0.0 ns	0.0 ns
Control	99.0	0.9	0.0	0.1	100	0.0	0.0	0.0
Nioro								
Treated	98.4 ns	0.9 ns	0.0 ns	0.7 ns	83.9*	15.7*	0.2 ns	0.2 ns
Control	99.0	0.2	0.0	0.8	57.3	42.7	0.0	0.0
2013								
Diourbel								
Treated	–[z]	–	–	–	99.0 ns	0.0 ns	0.0 ns	1.0 ns
Control	–	–	–	–	100	0.0	0.0	0.0
Nioro	–	–	–	–				
Treated	–	–	–	–	84.1 ns	15.9 ns	0.0 ns	0.0 ns
Control	–	–	–	–	76.3	23.7	0.0	0.0
2014								
Tambacounda								
Treated	97.0 ns	2.4 ns	0.1 ns	0.4 ns	86.9 ns	13.1 ns	0.0 ns	0.0 ns
Control	97.8	1.6	0.0	0.6	77.5	22.5	0.0	0.0
Nioro								
Treated	97.3 ns	1.3 ns	0.0 ns	1.4 ns	99.7 ns	0.2 ns	0.0 ns	0.1 ns
Control	96.0	3.0	0.0	1.0	100	0.0	0.0	0.0

[x] A, *Aspergillus aflatoxiformans*; L, *Aspergillus flavus* L-morphotype; P, *Aspergillus parasiticus*; T, *Aspergillus tamarii*.
[y] In each region, species/strain frequencies from treated samples with an asterisk (*) significantly differed from those found in its corresponding control treatment by Student's *t*-test ($\alpha = 0.05$); ns = non-significant.
[z] Soil samples were not collected before inoculation during 2013.

after storage. In several districts, in distinct years, none of the crop samples from treated fields contained >20 μg/kg aflatoxins either at harvest or after storage (Table 7). There was no consistent trend on percentage of samples with 4 to <20 μg/kg total aflatoxins among the specific treatment-crop-year combinations.

Discussion

This study sought to determine whether atoxigenic *A. flavus* strains native to Senegal applied in a biocontrol formulation are effective in reducing aflatoxin contamination in groundnut and maize grown in Senegal under farmers' field conditions. Crops from

Table 5. Combined frequencies of the four atoxigenic vegetative compatibility groups (VCGs) composing Aflasafe SN01 in soil, groundnut, and maize samples collected from control and Aflasafe SN01-treated fields in three districts of Senegal where efficacy trials of Aflasafe SN01 were conducted from 2010 to 2014[v]

	Aflasafe SN01 VCGs (%)[w]								
	2010		2011		2012		2013[x]	2014[y]	
District and treatment	Soil before inoculation	Grain at harvest	Soil before inoculation	Grain at harvest	Soil before inoculation	Grain at harvest	Grain at harvest	Soil before inoculation	Grain at harvest
Diourbel									
Treated	0.2 ns	61.8***	20.0 ns	56.7***	37.2*	42.2***	46.8***	–	–
Control	0.0	8.0	19.1	7.1	13.7	28.9	3.7	–	–
Nioro									
Treated	0.3 ns	69.4***	2.5 ns	72.9***	36.5 ns	62.7***	53.4***	20.9*	67.7***
Control	0.0	12.2	5.6	8.7	25.0	18.6	7.9	9.3	24.2
Tambacounda[z]									
Treated	–	–	–	–	–	–	–	37.2*	42.2***
Control	–	–	–	–	–	–	–	13.7	28.9

[v] Frequencies of the atoxigenic Aflasafe SN01 VCGs were determined using vegetative compatibility analyses based on nitrate nonutilizing mutants of all of the recovered *Aspergillus flavus* L-morphotype isolates.
[w] In each district and year, Aflasafe SN01 VCG frequencies from treated samples with one or three asterisks (*) significantly differed from those found in its corresponding control treatment by Student's *t* test (α = 0.05 and 0.001, respectively).
[x] Soil samples were not collected before application of SN01 during 2013.
[y] Maize was collected only in Nioro district during 2014; groundnuts from this district were not sampled during 2014. The Diourbel district was not sampled during 2014.
[z] The Tambacounda district was treated (groundnut) only during 2014.

Table 6. Total aflatoxin concentrations in freshly harvested and stored maize and groundnut sampled from control and Aflasafe SN01-treated fields in three districts of Senegal from 2010 to 2014[w]

			Total aflatoxin concentration									
			At harvest					After storage				
District	Crop	Treatment	Min	Max	Variance	Mean, μg/kg[x]	Reduction (%)[y]	Min	Max	Variance	Mean, μg/kg[x]	Reduction (%)[y]
2010												
Diourbel	Groundnut	Treated	0.0	7.3	4.8	1.4*	96.3	0.0	60.8	214	9.5**	77.0
Diourbel	Groundnut	Control	0.0	550.8	15,579	37.5		1.6	295.2	6,685	41.2	
Nioro	Groundnut	Treated	0.0	114.0	657	9.0 ns	58.3	0.0	27.7	53	5.0**	90.9
Nioro	Groundnut	Control	0.0	123.7	1,406	21.6		0.0	318.3	9,766	54.6	
2011												
Diourbel	Groundnut	Treated	0.0	29.6	44	2.6***	90.0	0.0	34.0	108	8.5 ns	83.9
Diourbel	Groundnut	Control	0.0	164.7	1,565	25.9		0.0	559.4	15,738	52.5	
Nioro	Groundnut	Treated	0.0	46.5	104	2.8***	97.6	0.0	62.6	217	9.9*	76.3
Nioro	Groundnut	Control	0.0	1,210.0	68,266	113.7		0.0	211.1	3,288	41.7	
2012												
Diourbel	Groundnut	Treated	0.0	21.1	31	3.7 ns	81.8	0.0	55.7	199	6.9 ns	80.7
Diourbel	Groundnut	Control	0.0	155.9	1,958	20.3		0.0	422.2	9,990	35.5	
Nioro	Groundnut	Treated	0.0	3.1	1	0.5**	98.2	0.0	60.4	236	6.8**	89.6
Nioro	Groundnut	Control	0.0	357.3	714.5	28.5		0.0	288.9	7,703	64.9	
2013												
Diourbel	Groundnut	Treated	0.0	9.3	5	0.7**	88.4	0.0	3.9	1	0.5*	96.2
Diourbel	Groundnut	Control	0.0	33.1	85	6.4		0.0	250.8	2,425	13.2	
Nioro	Groundnut	Treated	0.0	64.2	157	4.6*	84.9	0.0	43.8	73	2.5***	95.2
Nioro	Groundnut	Control	0.0	485.3	7,876	30.7		0.0	1,176.0	17,485	53.5	
2014												
Tambacounda[z]	Groundnut	Treated	0.0	430.0	7,903	26.5***	87.4	–	–	–	–	–
Tambacounda[z]	Groundnut	Control	1.7	2,136.0	170,479	210.0		–	–	–	–	
Nioro	Maize	Treated	0.0	0.0	0	0.0***	100	0.0	13.7	5	0.6***	93.9
Nioro	Maize	Control	0.8	48.1	65	4.8		0.0	86.4	274	10.1	

[w] Values in the mean column are the sum of aflatoxins B_1, B_2, G_1, and G_2.
[x] Means of aflatoxin values were compared independently between treated and control samples in each district and each year. Treated values with one, two, or three asterisks (*) significantly differed from its corresponding control treatment by Student's *t*-test (α = 0.05, 0.01, and 0.001, respectively).
[y] Percentage reduction was calculated for each district in each year as follows: ([mean of control − mean of Aflasafe SN01 treated]/mean of control) × 100.
[z] Groundnut samples from Tambacounda were not evaluated for aflatoxin concentrations after storage.

Aflasafe SN01-treated fields accumulated significantly less aflatoxins compared with crops from untreated fields. Indeed, most treated crops contained <4 μg/kg aflatoxins, low enough for entry into even stringent international food and feed markets. Low aflatoxin levels (58.3 to 100% less than control) and variance (53.3 to 100% less than control) in treated crops were associated with high incidences of Aflasafe SN01 VCGs both at harvest and throughout storage. This indicates that both aflatoxin accumulation and compositions of communities of aflatoxin-producing fungi were influenced by the use of Aflasafe SN01. Although effectiveness of biological control in reducing aflatoxin in groundnut has been reported in the United States (Dorner 2009) and Argentina (Alaniz Zanon et al. 2013), this is the first report of the efficacy of biocontrol for aflatoxin management in groundnut in Africa. Reduced variance in aflatoxin content is an advantage of atoxigenic genotype-based biocontrol not previously reported. Reduced variance suggests that values from aflatoxin assays are more reliable. This should result in treated crops with acceptable aflatoxin content at the port of origin having less likelihood of rejection when analyzed at the destination. Rejections at ports of destination carry both significant economic liability and potential for long-term loss of markets.

The Government of Senegal, through DPV, actively participated with IITA and the U.S. Department of Agriculture–Agricultural Research Service to develop and test the product Aflasafe SN01. The results from this collaborative study were used to prepare a dossier for registration of Aflasafe SN01 with Le Comité Sahélien des Pesticides of Comité Inter-Etate pour la Lutte contre la Sécheresse au Sahel (CSP/CILSS), the regulatory agency responsible for registering pesticides in 13 nations of the Sahel region, which includes Senegal. The unique SSR patterns of the four atoxigenic isolates served as the resource for identification of the active ingredients of Aflasafe SN01. In May 2016, CSP/CILSS approved the use of Aflasafe SN01 for aflatoxin mitigation in groundnut and maize throughout Senegal. Results presented in this report suggest that Aflasafe SN01 provides an important additional tool for aflatoxin management in groundnut and maize in Senegal.

In Senegal, aflatoxin contamination has severely impacted human health, income, and agricultural trade (Coursaget et al. 1993; Georges et al. 2016; Watson et al. 2015). Perennial contamination of groundnut with aflatoxins results in low proportions of crops meeting international standards. This particularly impacts smallholder farmers, because most of their income is obtained through the production of groundnut (Tankari 2017). Crops with aflatoxin concentrations below the maximum allowable levels of western markets receive premiums associated with market entry. Production of compliant groundnut in Senegal would allow exports to increase from 25,000 to 210,000 tons, with an increase in >$300 million U.S. dollars in annual revenue (Georges et al. 2016). However, this will only be possible with effective aflatoxin management and development of mechanisms to aggregate large quantities of groundnut with aflatoxin concentrations reliably below the maximum allowable level.

Aflatoxin producers become associated with both groundnut and maize during crop development, maturation, harvest, and storage. Therefore, aflatoxin management strategies need to be implemented long before harvest (Cotty and Mellon 2006). A strategy providing benefits from field to storage is the use of atoxigenic *A. flavus* strains as biocontrol agents to displace aflatoxin-producing genotypes in the field (Brown et al. 1991). Biocontrol formulations applied at the appropriate crop growth stage provide protection before, during, and after harvest and until crop consumption (Bandyopadhyay et al. 2016; Cotty 2006; Dorner 2004). The first atoxigenic biopesticides, developed in the United States, contain a single atoxigenic *A. flavus* genotype as the active ingredient (Cotty et al. 2007; Dorner 2004). In SSA, several biocontrol products have been developed under the trade name Aflasafe, each containing a mixture of four atoxigenic genotypes as active ingredients (Bandyopadhyay et al. 2016). In this study, four atoxigenic genotypes native to Senegal were selected for use as biocontrol agents to limit crop aflatoxin content. Those genotypes belong

Table 7. Percentage of samples within aflatoxin concentration categories in freshly harvested and stored groundnut/maize grains sampled from Aflasafe SN01-treated and control fields in three regions of Senegal from 2010 to 2014

			Percentage of samples in widely used total aflatoxin (μg/kg) categories[z]					
			At harvest			After storage		
Region	Crop	Treatment	<4	4 to <20	>20	<4	4 to <20	>20
2010								
Diourbel	Groundnut	Treated	83.3	16.7	0.0	55.6	33.3	11.1
Diourbel	Groundnut	Control	50.0	27.8	22.2	16.7	50.0	33.3
Nioro	Groundnut	Treated	55.6	38.8	5.6	61.1	33.3	5.6
Nioro	Groundnut	Control	44.7	38.6	16.7	27.8	38.9	33.3
2011								
Diourbel	Groundnut	Treated	80.0	15.0	5.0	45.0	35.0	20.0
Diourbel	Groundnut	Control	35.0	25.0	40.0	50.0	15.0	35.0
Nioro	Groundnut	Treated	90.0	5.0	5.0	45.0	40.0	15.0
Nioro	Groundnut	Control	25.0	20.0	55.0	20.0	45.0	35.0
2012								
Diourbel	Groundnut	Treated	70.6	23.5	5.9	70.6	17.6	11.8
Diourbel	Groundnut	Control	76.5	5.9	17.6	70.6	11.8	17.6
Nioro	Groundnut	Treated	100.0	0.0	0.0	80.9	4.8	14.3
Nioro	Groundnut	Control	61.9	14.3	23.8	42.9	9.5	47.6
2013								
Diourbel	Groundnut	Treated	93.8	6.2	0.0	92.0	8.0	0.0
Diourbel	Groundnut	Control	62.5	29.5	8.0	64.0	24.0	12.0
Nioro	Groundnut	Treated	82.0	10.0	8.0	90.0	4.0	6.0
Nioro	Groundnut	Control	78.0	2.0	20.0	56.0	6.0	38.0
2014								
Tambacounda	Groundnut	Treated	82.5	5.0	12.5	–	–	–
Tambacounda	Groundnut	Control	12.5	20.0	67.5	–	–	–
Nioro	Maize	Treated	100.0	0.0	0.0	92.0	8.0	0.0
Nioro	Maize	Control	76.7	18.6	4.7	48.0	40.0	12.0

[z] <4 μg/kg = below the European Union maximum total aflatoxin level for human consumption; <20 μg/kg = below the U.S. Food and Drug Administration action level for total aflatoxins in food; >20 μg/kg = universally considered unacceptable for human consumption. Category values were calculated independently by dividing the number of samples within a category by the total number of samples. The quotient was then multiplied by 100 to provide the percentage.

to VCGs with broad distribution across major agricultural areas in Senegal (Diedhiou et al. 2011). Detailed comparisons among the Aflasafe SN01 active ingredients and other atoxigenic genotypes of African and U.S. origins have been previously published (Adhikari et al. 2016). Briefly, all of the active ingredients in Aflasafe SN01 have multiple lesions in the aflatoxin biosynthesis gene cluster. Each of the lesions is sufficient to result in loss of aflatoxin-producing ability. This suggests that atoxigenicity has been conserved in all four of the active ingredient VCGs for sufficient periods to allow continued degeneration of the cluster. Genetic relationships among genotypes constituting Aflasafe SN01 and atoxigenic genotypes from the United States (*A. flavus* AF36 and Afla-Guard) and Nigeria (Aflasafe) are not related to geographical origin but rather, are related to similitudes in aflatoxin gene deletion patterns (Fig. 2) (Adhikari et al. 2016).

Intuitively, it would seem that the population of the genus *Aspergillus* in the field should increase with the application of Aflasafe SN01. In certain districts, in single years, higher fungal densities were detected in Aflasafe SN01-treated fields compared with control fields (Table 3). Thus, biocontrol applications in some cases will result in higher fungal densities, although of beneficial atoxigenic strains. However, in some cases, higher fungal densities were detected at harvest in crop samples from control fields (Table 3), but their aflatoxin content was low (Table 6). Atehnkeng et al. (2014) made similar observations in Nigeria and suggested that proportions of atoxigenic fungi may have been greater in fields with high *Aspergillus* densities but low aflatoxin content. Similarly, results from this study could also be explained by a relatively high proportion of atoxigenic strains—applied in neighboring fields—in those control fields. It is likely that, despite the isolation distance, the atoxigenic genotypes moved from the treated fields to control fields as reported for AF36 in cotton in Arizona (Bock et al. 2004; Cotty 1994b). Interfield dispersal of atoxigenic genotypes suggest that widespread use of Aflasafe SN01 over a large area is likely to provide area-wide benefits. Future studies should investigate aflatoxin-producing abilities of the fungi recovered from control fields. That would allow (i) clarification of aflatoxin-producing potentials of the isolates not identified as one of the applied active ingredients of Aflasafe SN01 and (ii) detection of additional atoxigenic genotypes for future use in developing new biocontrol products.

The *A. flavus* L-morphotype dominated all communities during the 5-year study. High *A. flavus* L-morphotype frequencies were detected in both treated and control soils and crops (Table 4). In control fields, high proportions were expected of both *A. aflatoxiformans*, a species native to West Africa (including Senegal) (Agbetiameh et al. 2018; Atehnkeng et al. 2008; Cardwell and Cotty 2002; Cotty and Cardwell 1999; Diedhiou et al. 2011; Donner et al. 2009; Frisvad et al. 2019; Probst et al. 2014), and *A. parasiticus*, a species commonly associated with groundnut cultivation in some regions (Horn et al. 1995; Kachapulula et al. 2017b; Klich 2002). However, these two species were detected only in certain years and restricted fields within the evaluated districts (Table 4). It is possible that *A. aflatoxiformans* and *A. parasiticus* in Senegal are not aggressive in infecting and colonizing groundnut. Host preference occurs within *Aspergillus* species (Mehl and Cotty 2013). However, despite their relatively low frequencies, both fungal types should be considered important etiologic agents of contamination based on their high aflatoxin-producing potential (Probst et al. 2014).

Community compositions of aflatoxin-producing fungi vary yearly within and among agroecologies (Ortega-Beltran et al. 2015). However, in this study, community compositions were relatively stable in both soils and groundnut from control fields in Diourbel and Nioro over multiple years (Table 4). Cropping systems and drought have been reported to influence populations of *A. flavus* and *A. parasiticus* (Horn et al. 1995). Influences of cropping systems and drought on stability of fungal communities warrant additional investigation. The active ingredients of Aflasafe SN01 are fungi endemic in Senegal, and as such, use of this biopesticide is not expected to pose new risks to nontarget species native to Senegal (Bandyopadhyay et al. 2016).

A. aflatoxiformans was relatively common in groundnut grains from control fields in Nioro but not in control grains of Diourbel (Table 4). Soil conditions in Diourbel do not seem to be conducive for *A. aflatoxiformans*. It is likely that the relatively high aflatoxin levels detected in groundnut in Diourbel were because of the presence of high proportions of aflatoxin-producing L-morphotype fungi (Table 6). In Nioro, *A. aflatoxiformans* occurred at relatively low frequencies in soils, but it was relatively common on groundnut grain but not maize grains. Perhaps this species is more common at depths greater than the 2-cm layer sampled in this study. Future studies should investigate whether *A. aflatoxiformans* composes greater proportions of *Aspergillus* communities resident at greater soil depth and whether environmental conditions, cropping systems, and/or resistance of the planted maize cultivars influence frequencies of this species in maize grains. Overall, results presented here indicate that members of the highly toxigenic *A. aflatoxiformans* should be expected to occur in most years in groundnut cultivated in Nioro unless Aflasafe SN01 is used.

Soil is an important reservoir of inoculum of *Aspergillus* section *Flavi* that infects crops. Frequencies of the atoxigenic Aflasafe SN01 active ingredients were low in soils before treatment in 2010 (Table 5). However, frequencies of active ingredient VCGs increased each year after Aflasafe SN01 application (range = 2.5 to 37.2%) (Table 5). Increased frequencies reflected carryover from previous year applications, but this was also observed in the control fields, which has been observed with AF36 in cotton (Cotty 1994b). In all cases, frequencies of the atoxigenic Aflasafe SN01 VCGs were significantly ($P < 0.001$) higher in crops from treated fields than in crops from control fields regardless of year, district, or crop (Table 5). High incidences of all of the active ingredients in treated crops indicate that all four VCGs are effective in displacing aflatoxin producers.

Substantial aflatoxin reductions in crops occurred in treated fields both at harvest (range = 58.3 to 100%) and throughout storage (range = 76.2 to 95.4%). Similar levels of reductions were reported in the United States and Argentina (Alaniz Zanon et al. 2013; Dorner 2009). Most crops from treated fields in most years had aflatoxin content meeting quality standards for sale in premium markets. Indeed, only a small portion of the crops from treated fields accumulated >20 μg/kg aflatoxins (Table 7), the aflatoxin threshold in Senegal. Combined with the lower concentration of aflatoxin, the concentration variance was also lower in treated crops compared with control crops, suggesting that aflatoxin values are more reliable for treated crops and as a result, have less risk of inaccurate analyses that may result in expenses of rejection after export or unwary exposure through ingestion (Table 6). Use of Aflasafe SN01 in maize and groundnut fields significantly increased farmers' chances to meet the stringent aflatoxin thresholds imposed by both local and international premium markets (Table 7), and it has the potential to revitalize the groundnut export sector, which has been severely affected for >30 years because of perennial crop aflatoxin contamination (Georges et al. 2016). However, it is important to note that use of Aflasafe SN01 did not completely eliminate aflatoxin concentrations in some of the treated crops. However, the reductions that were observed occurred in the absence of improved agronomic and storage practices. Use of appropriate agricultural, harvesting, storing, and processing practices would complement the use of Aflasafe SN01 and further decrease aflatoxin content throughout the value chain. Chronic exposure at even relatively low concentrations may have a significant impact on human health, particularly in children <5 years of age (Gong et al. 2008). Therefore, it is imperative to use all available appropriate technologies to decrease aflatoxin content to the lowest possible level.

Effectiveness of biocontrol in groundnut has been questioned (Njoroge 2018). Without providing empirical data, Njoroge (2018) argued that biological control is ineffective when drought prevails in groundnut. Although it is true that atoxigenic isolates would not sporulate on the carrier when there are long periods of drought, the fungi sporulate as soon as moist conditions return. Dorner (2009) demonstrated that biocontrol was particularly effective when aflatoxin conducive situation was promoted by drought stress. Although we did not collect water stress data in the trial sites, the groundnut basin, where the trials were conducted, is known to be drought prone (Clavel et al. 2005; Tschakert and Tappan 2004). Higher levels of aflatoxin reduction and lower variance in aflatoxin concentration in

treated fields compared with controls as determined by data from 536 trial sites during this 5-year study suggest that biocontrol is an effective tool, even in drought-prone areas. High aflatoxin reductions and low variance in crop aflatoxin concentration are a result of the high frequencies of Aflasafe SN01 VCGs. More research is required to (i) determine the relationship between the length of drought period and biocontrol performance, (ii) develop methods to improve product performance under extended periods of drought, and (iii) determine if Aflasafe SN01 application rates can be reduced or application made only during alternate years after a few years of continuous treatment.

There is a notion that sexual recombination can occur when atoxigenic biocontrol agents are applied in the field, and this could result in emergence of highly toxic strains (Ehrlich et al. 2015; Moore 2014; Moore et al. 2013; Olarte et al. 2012, 2015; Ouko et al. 2018). Atoxigenic strains used in biocontrol formulations are isolated from the same areas in which these are used; therefore, there has been ample opportunity for sexual recombination to occur under natural conditions. Well-planned studies examining fungal communities over decades in vast agricultural and nonagricultural areas have amply demonstrated that both toxigenic and atoxigenic genotypes—including atoxigenic genotypes used in biocontrol formulations—are highly stable in nature, that those communities are shaped predominantly by clonal reproduction and mutation, and that sexual recombination in nature is a process strongly restrained (Adhikari et al. 2016; Grubisha and Cotty 2010, 2015; Islam et al. 2018; Ortega-Beltran et al. 2016). Without providing empirical data, Ouko et al. (2018) hypothesized that sexual reproduction in Kenyan *Aspergillus* communities can occur, because both mating-type idiomorphs were detected in a set of *A. flavus* isolates. Yet, a very large population genetic study (Islam et al. 2018) in Kenya could not detect any sign of sexual recombination. Functionality of *mat* loci in aflatoxin-producing fungi has been questioned by several authors, including Dyer and O'Gorman (2012), Kwon-Chung and Sugui (2009), and Ouko et al. (2018). Sexual reproduction in aflatoxin-producing species has been demonstrated under laboratory fastidious conditions (Horn et al. 2009, 2011). Rather than demonstrations of sexuality in aflatoxin-producing species, it has been suggested that those are demonstrations of a process long lost in natural conditions (Kwon-Chung and Sugui 2009).

All four atoxigenic strains of Aflasafe SN01 are native and widely adapted to Senegalese agroecologies. Atoxigenic biocontrol products containing fungi exotic to Senegal should not be considered for use in this nation. Native atoxigenic strains locally adapted to target crops in Senegal have a greater chance to dominate treated areas and establish long-term, safe *Aspergillus* communities (Mehl et al. 2012; Probst et al. 2011). The multistrain biocontrol product Aflasafe SN01 has the potential to promote stable, safe *Aspergillus* communities tolerant to biotic and abiotic changes that may occur within or among cropping seasons. A similar biocontrol product utilizing multiple atoxigenic strains in Nigeria has been reported to be successful in promoting *Aspergillus* communities with low aflatoxin-producing potentials (Atehnkeng et al. 2014, 2016).

Large-scale use of Aflasafe SN01 would provide substantial benefits to trade and human health in Senegal. Portions of safe crops from treated fields would be consumed by farmers and their families, whereas the remainder would enter both informal and organized markets. This would result in reduction of human exposure to dangerous aflatoxin concentrations (Watson et al. 2015). Additionally, a large proportion of groundnut harvested from Aflasafe SN01-treated fields complied with aflatoxin standards, furthering trade opportunities and income generation for farmers (Table 7). To enable large-scale use of Aflasafe SN01 after its registration, IITA has licensed manufacturing and distribution responsibilities of Aflasafe SN01 to BAMTAARE SA, a private company in Senegal that works with >70,000 smallholder farmers. The technology will benefit Senegalese farmers, particularly smallholder farmers, and the Senegalese population in general.

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