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 directly related to the levels 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.

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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 coinfected 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 throughput storage and (ii) increased frequencies of Aflasafe SN01 atoxigenic genotypes in treated fields. After approval 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 (Cailloret 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 haplotype frequencies 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 SplitsTree4 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 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% V8 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 falciform colonies 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-Hegilton 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...
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. aflatuitavorans, A. parasiticus, or Aspergillus tamarii) based on colony characteristics and spore ornamentation (Cotty 1999; 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 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, y) 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, α = 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 Arachidiere, 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 to controls.

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

| Crop and treatment | 2010 Diourbel | 2010 Niayes | 2011 Diourbel | 2011 Niayes | 2012 Diourbel | 2012 Niayes | 2013 Diourbel | 2013 Niayes | 2014 Tambacounda | 2014 Niayes |
|--------------------|---------------|-------------|---------------|-------------|---------------|-------------|---------------|-------------|----------------|-------------|
| Groundnut          |               |             |               |             |               |             |               |             |                 |             |
| Treated            | 18            | 18          | 20            | 20          | 17            | 21          | 18            | 42          | 50             | –           |
| Control            | 18            | 18          | 20            | 20          | 17            | 21          | 18            | 42          | 50             | –           |
| Maize              |               |             |               |             |               |             |               |             |                 |             |
| Treated            | –             | –           | –             | –           | –             | –           | –             | –           | 44             |             |
| Control            | –             | –           | –             | –           | –             | –           | –             | –           | 44             |             |

* In all fields, soil samples were collected before treatment, except in 2013, and crop samples at harvest.

* Efficacy trials not conducted.
Aflatoxin concentrations were generally >20 μg/kg in all regions, crops, and years. Frequencies of Aflasafe SN01 VCGs were significantly (P < 0.05) higher in all treated fields in comparison with control fields, combined recovery of Aflasafe SN01 VCGs was significantly (P > 0.0001) higher in all treated fields in comparison with control fields, and aflatoxin reductions ranged from 86.8% fewer aflatoxins than crops in 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% from control fields both at harvest and after storage (Table 7).

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.

| Product | Active ingredients | AF28 | AF38 | AF39 | AF42 | AF53 | AF54 | AF16 | 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 | NRRRL21882 | 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 | M2-11 | 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  |

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

| District and treatment | 2010          | 2011          | 2012          | 2013*         | 2014*         |
|------------------------|---------------|---------------|---------------|---------------|---------------|
|                        | Soil before inoculation | Grain at harvest | Soil before inoculation | 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      |
| Control                | 2,120         | 11,576        | 431           | 22,385        | 959           | 26,717         |
| Nioro                  |               |               |               |               |               |               |
| Treated                | 576 ns        | 58,124*       | 434 ns        | 129,090*      | 1,054 ns      | 20,763 ns      |
| Control                | 103           | 6,908         | 326           | 11,325        | 476           | 9,398          |
| Tambacounda            |               |               |               |               |               |               |
| Treated                | –             | –             | –             | –             | –             | –              |
| Control                | –             | –             | –             | –             | –             | –              |

\* 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 (α = 0.05). ns = non-significant.

\* Soil samples were not collected before application of Aflasafe SN01 during 2013.

\* 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.

\* Efficacy trials not conducted.

\* 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

| Year, district, and treatment | Soil before inoculation | Grain at harvest |
|------------------------------|------------------------|------------------|
|                              | 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                      | –   | –   | –   | –   | 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   |

\* A, *Aspergillus aflatoxiformans*; L, *Aspergillus flavus* L-morphotype; P, *Aspergillus parasiticus*; T, *Aspergillus tamarii*.

\* 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 (α = 0.05); ns = non-significant.

\* 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

| District and treatment | 2010 | 2011 | 2012 | 2013 | 2014 |
|------------------------|------|------|------|------|------|
|                        | Soil before inoculation | Grain at harvest | Soil before inoculation | Grain at harvest | Soil before inoculation | Grain at harvest |
| **Aflasafe SN01 VCGs (%)** |      |      |      |      |      |
| **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**** |
| Control                | 0.0 | 12.2 | 5.6 | 8.7 | 25.0 | 18.6 | 7.9 |
| **Tambacounda** Treated | – | – | – | – | – | 37.2* | 42.2*** |
| Control                | – | – | – | – | – | 13.7 | 28.9 |

Notes:
- 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.
- 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).
- Soil samples were not collected before application of SN01 during 2013.
- 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.
- 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

| District | Crop | Treatment | At harvest | After storage |
|---------|------|-----------|------------|--------------|
|         |      |           | Mean, μg/kg | Reduction (%) | Min | Max | Variance | Mean, μg/kg | Reduction (%) |
|         |      |           |            |              |      |     |          |            |              |
| 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 | 0.0 | 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 | Groundnut | Treated | 0.0 | 430.0 | 7,903 | 26.5*** | 87.4 | – | – | – | – |
|         | Tambacounda | 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 |

Notes:
- Values in the mean column are the sum of aflatoxins B1, B2, G1, and G2.
- 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 those found in its corresponding control treatment by Student’s *t*-test (*α* = 0.05, 0.01, and 0.001, respectively).
- Percentage reduction was calculated for each district in each year as follows: ((mean of control – mean of Aflasafe SN01 treated)/mean of control) × 100.
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 (Alamiz 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 ITA 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 Pes- cicides of Comité Inter-Etat 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

| Region     | Crop   | Treatment | At harvest | After storage |
|------------|--------|-----------|------------|--------------|
|            |        |           | <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 | 4.8 | 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 |

* <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. aflatuxiformans, a species native to West Africa (including Senegal) (Agbetiaemeh 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. aflatuxiformans 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 non-target species native to Senegal (Bandyopadhyay et al. 2016).

A. aflatuxiformans 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. aflatuxiformans. 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. aflatuxiformans 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. aflatuxiformans 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. aflatuxiformans 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 (Alainz 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 (Clavel et al. 2005; Tschakert and Tappan 2004). Substantial aflatoxin reductions were observed in maize, groundnut, cotton, and pigeonpea and in other atoxigenic genotypes of African and U.S. origins have been previously published (Adhikari et al. 2016). Therefore, it is imperative to use all available appropriate technologies to 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.

Effective use of biocontrol has been questioned (Njorge 2018). Without providing empirical data, Njorge (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) argued 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; Oko 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, Oko 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 mating loci in aflatoxin-producing fungi has been questioned by several authors, including Dyer and O’Gorman (2012), Kwon-Chung and Sugui (2009), and Oko 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 these 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 coming from fungal exotics to Senegal should not be a tool 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, IFIA 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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