Aflasafe SN01 is the First Biocontrol Product Approved for Aflatoxin Mitigation in Two Nations, Senegal and The Gambia

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Abstract

Aflatoxin contamination is caused by *Aspergillus flavus* and closely related fungi. In The Gambia, aflatoxin contamination of groundnut and maize, two staple and economically important crops, is common. Groundnut and maize consumers are chronically exposed to aflatoxins, sometimes at alarming levels, and this has severe consequences on their health and productivity. Aflatoxin contamination also impedes commercialization in local and international premium markets. In neighboring Senegal, an aflatoxin biocontrol product containing four atoxigenic isolates of *A. flavus*, Aflasafe SN01, has been registered and is approved for commercial use in groundnut and maize. We detected that the four genotypes composing Aflasafe SN01 are also native to The Gambia. The biocontrol product was tested during two years in 129 maize and groundnut fields and compared with corresponding untreated fields cropped by

smallholder farmers in The Gambia. Treated crops contained up to 100% less aflatoxins than untreated crops. A large portion of the crops could have been commercialized in premium markets due to the low aflatoxin content (in many cases no detectable aflatoxins), both at harvest and after storage. Substantial aflatoxin reductions were also achieved when commercially produced groundnut received treatment. Here we report for the first time the use and effectiveness of an aflatoxin biocontrol product registered for use in two nations. With the current scale-out and -up efforts of Aflasafe SN01, a large number of farmers, consumers, and traders in The Gambia and Senegal will obtain health, income, and trade benefits.

Keywords: aflatoxin biocontrol, atoxigenic isolates, postharvest benefits

In tropical and subtropical areas, aflatoxins contaminate several staple crops, including maize and groundnut, during crop production

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Funding: Funding support of the United States Department of Agriculture, Foreign Agricultural Service, the Bill & Melinda Gates Foundation (OPP1007117, OPP1133356), and the CGIAR Research Program on Agriculture for Nutrition and Health (A4NH) by CGIAR Trust Fund contributors (<https://www.cgiar.org/research/>) is gratefully acknowledged.

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The authors receive no direct financial benefit from the manufacturing and marketing of aflatoxin biocontrol products mentioned in this article. The Aflasafe name is a Trademark of the International Institute of Tropical Agriculture (IITA). In the past, IITA manufactured Aflasafe for use in Nigeria, Senegal, Kenya, Burkina Faso, The Gambia, and Ghana. Manufacturing and distribution responsibilities have been licensed to private or public sector entities in a few African nations. IITA charges a small licensing fee to manufacturers for use of the Aflasafe name and cost associated with technology transfer and technical backstopping. L. A. Senghor, A. Ortega-Beltran, J. Atehnkeng, and R. Bandyopadhyay are employed by IITA.

Accepted for publication 15 December 2020.

and postproduction stages (Udomkun et al. 2017a). Aflatoxins are highly toxic and carcinogenic mycotoxins produced by certain members of *Aspergillus* section Flavi (Amaike and Keller 2011; Frisvad et al. 2019). Aflatoxin-producing fungi infect crops before, during, and after harvest and can produce aflatoxins during all these stages if conducive conditions for toxin formation occur (Kachapulula et al. 2017a; Seetha et al. 2017). Aflatoxin contamination results in significant health, developmental, trade, and economic problems in the affected regions/nations (Nishimwe et al. 2019; Wu 2015). The aflatoxin problem is frequent in West African nations. In The Gambia, only a few studies report aflatoxin in crops/foods (Hudson et al. 1992, Jallow et al. 2019; Xu et al. 2017) and none at a nationwide household scale. However, in The Gambia, high human aflatoxin exposure is common (Groopman et al. 1992; Kirk et al. 2006; Kuniholm et al. 2008; Wild et al. 1992), including among children and pregnant mothers (Castelino et al. 2014; Turner et al. 2000, 2003, 2007; Zarba et al. 1992). Exposure at an early life stage may impede normal child development and proper cognitive functions (JECFA 2018). Aflatoxin management strategies to reduce contamination throughout the crop value chains and reduce human exposure are urgently needed for implementation across The Gambia.

The Gambia Standards Bureau has set up the standard GAMS CAC/RCP 55 – 2004, a code of good practice for the prevention and reduction of aflatoxin contamination in groundnut (<https://tgsb.gm/published-standards>). This standard is based on maximum aflatoxin limits set by the Codex Alimentarius Commission (GAMX Codex Standard 200-1915), 15 parts per billion (ppb), for groundnut to be processed (PACA 2019). However, monitoring and enforcing aflatoxin regulations in The Gambia, and across sub-Saharan Africa (SSA), is challenged by several technical, policy, and institutional barriers (Matumba et al. 2017; PACA 2019). Groundnut consignments from The Gambia to the European Union, even as less lucrative feed, are frequently notified for rejection by the Rapid Alert System for Food and Feed for having unacceptable aflatoxin levels (RASFF 2019). Improved capacity to manage aflatoxins would significantly increase export potential of groundnut even for the more lucrative human food market.

The major aflatoxin producer across the globe is *A. flavus*. This species can be subdivided into two distinct morphotypes, the L and the S, based on morphological, physiological, and genetic criteria (Cotty 1989; Probst et al. 2014). Both morphotypes can produce B aflatoxins. However, in the L morphotype, genotypes unable to produce aflatoxins (i.e., atoxigenic) are common and contain genetic defects in one or more of the genes necessary for aflatoxin biosynthesis (Chang et al. 2012; Donner et al. 2010). The S morphotype consistently produces high aflatoxin levels (Cotty et al. 2008). In West Africa, fungi belonging to the B-aflatoxin-producing S morphotype of *A. flavus* have not been detected. In contrast, a group of fungi relatively common across the subregion resembles the S morphotype but produces both B and G aflatoxins (Atehnkeng et al. 2008; Cotty and Cardwell 1999; Diedhiou et al. 2011; Ezekiel et al. 2014). That group was named as unknown taxon S_{BG} and may be any of the recently described species *A. aflatoxiformans*, *A. austwickii*, *A. cerealis*, *A. minisclerotigenes*, or unnamed taxa (Frisvad et al. 2019; Probst et al. 2014; Singh and Cotty 2019). In this paper, as in previous ones (Agbetiamah et al. 2019; Ezekiel et al. 2019), we use the term 'S_{BG} strains' for all fungi with S morphotype and producing both B and G aflatoxins. Each of the fungi mentioned above can be subdivided into vegetative compatibility groups (VCGs) (Bayman and Cotty 1991, 1993; Leslie 1993). Members of a VCG are more closely related among themselves than to members of other VCGs and there is little genetic variation within a VCG (Grubisha and Cotty 2010, 2015; Ortega-Beltran et al. 2016).

Many practices can reduce crop aflatoxin content at different stages of the crop value chain (Aoun et al. 2020; Mahuku et al. 2019; Seetha et al. 2017; Waliyar et al. 2015; Xu et al. 2017). However, when each practice is used in isolation, aflatoxin contamination often occurs above safe, permissible levels, particularly in tropical and subtropical regions (Ayalew et al. 2017). An effective, practical technology that provides protection to crops across the value chain is the use of atoxigenic isolates of *A. flavus* as biocontrol agents (Cotty 2006; Dorner 2004; Mehl et al. 2012). Atoxigenic isolates applied in the field, at the right stage of crop production, creates a founding population (Ortega-Beltran et al. 2020) that displace aflatoxin producers, and thus less aflatoxins accumulate in treated crops. The technology was developed by the United States Department of Agriculture – Agricultural Research Service (USDA-ARS) for use in cottonseed production in the U.S. (Cotty et al. 2007). It has been adapted for use in maize, groundnut, pistachio, almond, and figs produced in the U.S. (Dorner 2009; Doster et al. 2014; Ortega-Beltran et al. 2019). Production of highly lucrative crops in aflatoxin-prone areas of the U.S. could not be possible without the protection provided by an aflatoxin management system centered on biocontrol (Cotty et al. 2007; Doster et al. 2014; Ortega-Beltran and Bandyopadhyay 2019).

The aflatoxin biocontrol technology has been adapted and improved for use in SSA by the International Institute of Tropical Agriculture (IITA) and USDA-ARS in collaboration with several national institutions and international partners (Bandyopadhyay et al. 2016; Schreurs et al. 2019). Several atoxigenic biocontrol products under the trade name Aflasafe have been developed. They are now available for commercial use in Nigeria, Kenya, Senegal, Ghana, Burkina Faso, Tanzania, Zambia, Malawi, and Mozambique (Moral et al. 2020). Each product contains as active ingredient four atoxigenic isolates of *A. flavus* native to the target country. The atoxigenic isolates belong to atoxigenic African *A. flavus* VCGs (AAVs), and some products contain AAVs present in one or more countries (Agbetiamah et al. 2019; Moral et al. 2020). When applied at the right crop stage and at the recommended dose, biocontrol-treated crops typically contain >80% less (sometimes 100%) aflatoxins compared with neighboring, untreated crops, and most treated crops can meet the most rigid aflatoxin regulatory standards (Agbetiamah et al. 2020; Bandyopadhyay et al. 2019; Ezekiel et al. 2019; Senghor et al. 2020).

Native and wide distribution nature of the active ingredients are important considerations in the selection of isolates composing each biocontrol product targeted for any country (Bandyopadhyay et al. 2016; Mehl et al. 2012). Aflasafe SN01, the product developed for

use in Senegal, is registered by the Comité Sahélien des Pesticides (CSP) of the Comité Inter-Etats pour la Lutte contre la Sécheresse au Sahel (CILSS) for use in maize and groundnut (Bandyopadhyay et al. 2016; Senghor et al. 2020). The four atoxigenic AAVs composing the product are distributed across major agricultural areas of Senegal. Thus, it is highly likely that those genotypes could co-occur in the neighboring country, The Gambia. If the four Aflasafe SN01 AAVs occur in The Gambia, it would be plausible to pursue the use of the product in that country because the genotypes would be adapted to its environmental and cropping conditions. Superior adaptation may increase the chances of success in reducing aflatoxin contamination under field conditions (Mehl et al. 2012). Noteworthy, registration of a biopesticide with CSP/CILSS allows the use of such product in all other CILSS countries, including The Gambia. However, finding Aflasafe SN01 AAVs in the target intervention area and testing the product across The Gambia are necessary to ensure that the active ingredient AAVs will be effective (Bandyopadhyay et al. 2016).

The objectives of this study were to determine i) aflatoxin prevalence in groundnut across The Gambia, ii) compositions of *Aspergillus* section Flavi communities in The Gambia, iii) presence/absence of Aflasafe SN01 AAVs in those communities, and iv) effectiveness of Aflasafe SN01 in reducing aflatoxin in groundnut and maize. This study laid the foundation for using the same biocontrol product (Aflasafe SN01) commercially to manage aflatoxins in The Gambia in addition to Senegal.

Materials and Methods

Sample collection and preparation. Groundnut samples were collected during January 2013 across seven regions of The Gambia: Central River North (CRN), Central River South (CRS), Lower River (LR), North Bank (NB), Upper River North (URN), Upper River South (URS), and West Coast (WC) (Fig. 1). Those are the major groundnut-producing regions of the country. Four villages were visited per region, and five groundnut samples (unshelled pods, ~1 kg) were collected from farmer stores (140 samples, Table 1). Groundnut in The Gambia is harvested during October to November. By the time of collection, the samples had remained in the farmers' store for 2 to 3 months. The samples were transported by road to Dakar, Senegal, and the quarantine authority of La Direction de Protection Végétaux (DPV) of Senegal provided a phytosanitary permit to send the samples to IITA's Pathology and Mycotoxin Unit in Ibadan, Nigeria, for laboratory analyses. Nigeria Agricultural Quarantine Service (NAQS) provided an import permit. Pods were shelled and kernels (approx. 750 g) were milled using a laboratory blender (Waring Commercial, Springfield, MO) for 30 s in a 250-ml stainless steel blending jar (MC-2) and stored at 4°C before aflatoxin and microbial analyses. The blending jar was thoroughly washed with 80% ethanol after milling each sample to prevent cross-contamination of aflatoxins and microorganisms among samples.

Aflatoxin analysis, baseline study, and effectiveness trials. For each milled groundnut sample (approx. 750 g), a 20 g subsample was randomly taken. The subsamples were combined with 100 ml 80% methanol (Dorner and Cole 1993). The resulting suspensions were shaken on a Roto-Shake Genie (Scientific Industries, Bohemia, NY) for 30 min at 400 rpm. Then, suspensions were filtered through Whatman No. 1 filter paper (Whatman Intl. Ltd., Maidstone, England). Aflatoxins from the filtrates were then extracted as previously described (Agbetiamah et al. 2018; Atehnkeng et al. 2008). Aflatoxins were quantified using a scanning densitometer (CAMAG TLC Scanner 3) and quantification software (winCATS 1.4.2, Camag, AG, Muttens, Switzerland) as described previously (Cotty and Cardwell 1999; Probst and Cotty 2012). The limit of detection was 1 ppb, while the recovery percentage was >85%.

Fungal isolation, identification, and densities. Compositions of fungal communities associated with groundnut in The Gambia were determined by identifying *Aspergillus* section Flavi fungi and

characterizing them into their corresponding species (Table 2). For this, the dilution plate technique on modified Rose Bengal agar was used (Cotty 1994), and a total of 12 *Aspergillus* section *Flavi* isolates were recovered from each sample as previously described (Agbetiamah et al. 2018; Atehnkeng et al. 2008). *Aspergillus* section *Flavi* fungi were identified based on their colony characteristics, spore ornamentation, and aflatoxin-production profile (Cotty 1989; Cotty and Cardwell 1999; Klich and Pitt 1988). Isolates were saved as agar plugs (3 mm diameter) of sporulating cultures in 4 ml vials containing 2 ml sterile distilled water and maintained at room temperature until used in other studies. Population density of *Aspergillus* section *Flavi* fungi was calculated as colony-forming units (CFU) per g of sample as per methods described previously (Agbetiamah et al. 2018; Atehnkeng et al. 2008).

Aflatoxin-producing potential of the recovered fungi. Each of the 1,608 *Aspergillus* section *Flavi* recovered isolates was evaluated for their ability to produce aflatoxins (Table 3) in sterile, dead autoclaved maize kernels (Agbetiamah et al. 2018;

Probst and Cotty 2012). Aflatoxins were extracted from maize fermentations and quantified as described above, except that 70% methanol was used instead of 80%. Isolates that did not produce aflatoxins on maize fermentations were classified as atoxigenic.

Vegetative compatibility analyses. All the recovered *A. flavus* L morphotype isolates were subjected to vegetative compatibility analyses (VCA) to determine whether they belong to any of the four AAVs to which the isolates composing Aflasafe SN01 belong (Table 4). Nitrate-nonutilizing (*nit*) mutants were generated as previously described (Atehnkeng et al. 2014, 2016). Tester pairs of AAVs in Aflasafe SN01 were previously generated (Senghor et al. 2020). VCA and assignment of isolates into one of the four AAVs composing the biocontrol product were conducted as previously described (Atehnkeng et al. 2014; Grubisha and Cotty 2010).

The biocontrol product and its manufacturing. Aflasafe SN01 was produced in IITA-Ibadan (Nigeria) in the Aflasafe

Fig. 1. Map of The Gambia showing sample collection sites for baseline aflatoxin analysis in groundnut, January 2013. In each region, four villages were visited, and five samples were collected from each village.

Table 1. Aflatoxin concentration in groundnut samples collected in seven regions of The Gambia in 2013

Region	n	B aflatoxins (ppb)				G aflatoxins (ppb)			
		Min ^y	Max	Mean ^z	Variance	Min	Max	Mean	Variance
Central River North	20	ND	570	70 ab	19,137	ND	588	33 ab	17,182
Central River South	20	ND	246	37 ab	4,310	ND	23	2 ab	29
Lower River	20	ND	19	2 b	25	ND	11	0.5 b	6
North Bank	20	ND	357	79 ab	15,417	ND	206	23 ab	8,898
Upper River North	20	ND	44	4 b	98	ND	14	0.7 ab	10
Upper River South	20	ND	208	16 b	2,145	ND	5	0.2 b	1
West Coast	20	ND	1,411	195 a	166,085	ND	578	73 a	21,128
LSD				0.5				0.4	

^y ND: not detected. Limit of detection = 1 ppb. Aflatoxins were extracted as previously described (Agbetiamah et al. 2018; Atehnkeng et al. 2008) and quantified with a scanning densitometer as previously described (Cotty and Cardwell 1999; Probst and Cotty 2012).

^z Means separation of log-transformed aflatoxin concentrations was conducted using Fisher's protected LSD test ($\alpha = 0.05$). Values followed by different letters differ significantly.

Manufacturing Plant using an industrial process previously described (Agbetiameh et al. 2020). Briefly, to prepare the biocontrol product, sterile sorghum grain was coated with a suspension containing spores of the four atoxigenic isolates, a blue food colorant to differentiate the product from regular sorghum, and a polymer that aids in the coating process. The quality of the product (purity, sporulation capacity, and composition of the active ingredient fungi) was determined as previously described (Senghor et al. 2020). The finished formulated product was placed in 2.5 kg polyethylene bags, sealed, and transported (by sea-freight) to Dakar, Senegal, under appropriate export permit from NAQS and import permit from DPV Senegal. Then, the product was transported by road from Senegal to The Gambia.

Field plots and biocontrol application for effectiveness trials. The biocontrol product was applied in maize and groundnut fields in four regions of The Gambia during the 2014 and 2015 cropping seasons (Table 5) in collaboration with members of farmers' associations. All farmers voluntarily consented to conduct the effectiveness trials. In 2014, maize fields were treated in CRN, and groundnut

fields were treated in CRN, NB, and WC. In 2015, maize fields were treated in both CRN and LR, and in groundnut fields in CRN, NB, and WC. The number of biocontrol-treated and untreated fields is given in Table 5.

During this part of the research, farmers were advised to grow their crops according to their traditional agronomic practices to test the value of biocontrol as a standalone management strategy in the real world. Groundnut farmers planted either 28-206 or 73-33, two of the most common groundnut varieties in The Gambia. The former is a late-maturity variety (125 day growing cycle; flowering time around 45 days after planting) while the latter is an intermediate-maturing variety (115 day growing cycle; flowering time around 40 days after planting) (ICRISAT 1990). Maize farmers planted diverse improved or landrace varieties with flowering time around 60 to 75 days after planting. In general, both crops were planted after the first rains during the first two weeks of July and harvested during late October to the first half of November. In general, farmers' fields were weeded by hand and animal-drawn hoe, top-dressed with urea

Table 2. Frequencies and combined densities of *Aspergillus* species associated with groundnut collected in seven regions of The Gambia in 2013

Region	n	<i>Aspergillus</i> species distribution (%) ^y					CFU/g ^z
		L	S _B	S _{BG}	P	T	
Central River North	240	83.8 ab	0.4 a	15.4 bc	0.0 b	0.4 a	31,971 b
Central River South	240	85.0 a	0.0 a	12.9 c	2.1 a	0.0 a	97,791 ab
Lower River	204	71.1 ab	0.0 a	28.9 bc	0.0 b	0.0 a	1,089 b
North Bank	216	58.3 bc	0.5 a	41.2 ab	0.0 b	0.0 a	247,589 ab
Upper River North	240	80.8 ab	0.0 a	19.2 bc	0.0 b	0.0 a	1,428 b
Upper River South	228	81.6 ab	0.0 a	18.4 bc	0.0 b	0.0 a	4,457 b
West Coast	240	42.9 c	0.0 a	57.1 a	0.0 b	0.0 a	246,425 a
Average		72.0	0.2	27.4	0.3	0.1	90,107

^y L: *A. flavus* L morphotype; S_B: fungi with S morphotype producing only B aflatoxins; S_{BG}: fungi with S morphotype producing both B and G aflatoxins; P: *A. parasiticus*; T: *A. tamarii*. Means separation of *Aspergillus* species frequencies was conducted using Fisher's protected LSD test ($\alpha = 0.05$). Values followed by different letters differ significantly.

^z Colony forming units per gram.

Table 3. Aflatoxin-producing potentials of aflatoxigenic fungi associated with groundnut collected in seven regions of The Gambia in 2013

Region	B aflatoxins (ppb)			G aflatoxins (ppb)		
	n ^y	Range	Mean ^z	n	Range	Mean
Central River North	209	8–7,842	1,638 bc	38	308–4,609	1,878 abc
Central River South	213	7–9,932	1,587 bc	36	61–4,444	1,379 c
Lower River	185	5–8,153	2,012 ab	59	98–5,110	2,171 ab
North Bank	196	16–6,289	2,019 ab	89	44–6,764	1,907 abc
Upper River North	220	7–9,478	2,373 a	46	98–14,101	2,365 a
Upper River South	183	6–10,631	1,564 c	42	319–3,744	1,564 bc
West Coast	233	15–8,989	2,431 a	137	155–4,891	2,031 abc

^y Number of *Aspergillus* isolates, regardless of corresponding species, producing the indicated toxins in each region.

^z Means separation of mean aflatoxin production potentials in each region was conducted using Fisher's protected LSD test ($\alpha = 0.05$). Values followed by different letters differ significantly.

Table 4. Number of atoxigenic *Aspergillus flavus* L morphotype isolates detected in seven regions of The Gambia and number of isolates belonging to the atoxigenic African *A. flavus* vegetative compatibility groups (AAVs) to which the active ingredient fungi of Aflasafe SN01 belong

Region	Number of atoxigenic L morphotype isolates	Members of Aflasafe SN01 AAVs ^z			
		SS19-14	MS14-19	M2-7	M21-11
Central River North	31	–	1	1	–
Central River South	27	1	–	–	–
Lower River	19	–	–	–	–
North Bank	20	–	–	–	–
Upper River North	20	–	–	1	–
Upper River South	45	4	–	2	1
West Coast	7	–	–	1	–

^z Frequencies of the atoxigenic Aflasafe SN01 AAVs were determined using vegetative compatibility analyses based on nitrate nonutilizing mutants of all the recovered *A. flavus* L morphotype isolates.

(for maize) or a complex fertilizer (for groundnut and maize), and earthed-up (i.e., piling up soil around the base of the plants) before application of the biocontrol product to avoid burying it. No other agronomic interventions were made.

Farmers were trained on biocontrol product broadcasting techniques as described by Senghor et al. (2020). The product was broadcasted by hand at a rate of 10 kg/ha. Each year, biocontrol application was done 2 to 3 weeks before flowering for maize and 30 to 35 days after planting for groundnut, depending on the crop and variety used. For each treated field, a neighboring field, managed by the same farmer and planted with the same variety, at least 500 m apart was selected as the corresponding untreated field. The physical separation reduced the movement of the atoxigenic isolates into untreated fields and crops (Bock et al. 2004). Field sizes ranged from 0.5 to 2 ha. Groundnut and maize cultivars varied from region to region. Production of crops in all fields was dependent on rainfalls, which were erratic.

Soil and crop sampling of effectiveness trials. Before biocontrol treatment, soil samples (~150 g) were collected by subsampling each field across transects from three random (40 to 50 subsamples) locations to a depth of 2 cm (Cotty 1997). Samples were air-dried and then sent to IITA-Ibadan under import/export permits as described above. After arrival, samples were dried in a forced-air oven (2 days, 50°C) and then transferred to a biosafety cabinet where soil clods were eliminated with a hammer and manually homogenized within plastic bags.

Crops were harvested immediately after reaching physiological maturity to determine the influences of biocontrol application on both fungal community structure (soil and kernels) and the effectiveness in reducing aflatoxin contamination in treated crops compared with untreated crops. From each field, a ~2 kg sample was collected. In 2014, an additional ~1 kg sample was collected 3 months after harvest, from the store of each farmer to evaluate the influence of storage on aflatoxin contamination in both treated and untreated crops. All samples were transferred to DPV Senegal Plant Pathology laboratory, milled using a blender as above, and then sent to IITA-Ibadan for aflatoxin and microbiological analyses.

Fungal identification in biocontrol effectiveness trials. Fungi belonging to *Aspergillus* section Flavi were recovered and assigned to their corresponding species as described above except that instead of selecting 12 isolates per sample, 16 isolates were selected. Isolates were saved as described above. Mutant generation, mutant saving, and VCA were conducted as described above.

Field plots and biocontrol application in commercial trials. In 2018, the biocontrol product was applied in commercial groundnut fields of CRN, NB, and WC. There were 15 treated fields in each of the three regions, and for each treated field there was an untreated control field located at least 500 m apart. Treated and corresponding untreated fields were managed by the same farmer and planted with the same variety. The size of the fields ranged from 1 to 6 ha. The biocontrol product was produced, transported to The Gambia, and applied as described above.

In-situ aflatoxin quantification in commercial crops. Aflatoxin extraction and quantification were conducted in the farmer fields after harvest. For this, a GIPSA-approved Neogen Reveal Q+ for Aflatoxin kit (Neogen Corp., Lansing, MI) was used. Briefly, 2 kg of groundnut was randomly collected, milled as described above, and a 5-g subsample was transferred into a 500 ml media bottle, and 100 ml 65% ethanol was added. The mixture was shaken by hand for 3 min and allowed to settle for 3 min. Then the mixture was filtered through Whatman No. 1 filter paper into a Tri-Pour beaker. Thereafter, 500 ml of sample diluent was transferred to a sample cup and 100 ml of sample filtrate was added. A 100 ml aliquot of diluted sample was transferred into a new cup and mixed thoroughly and mixed by aspiration. A lateral flow strip was placed in the sample cup for 6 min, and then the strip was read on a Neogene AccuScan Gold Reader. The limit of detection was 1 ppb total aflatoxin.

Data analysis. Data on aflatoxin concentration, *Aspergillus* species distribution, CFU/g, and incidences of Aflasafe SN01 AAVs (response variable, x) were transformed using the equation $y = \log_{10}(1+x)$ to stabilize the variance. For the baseline study, means were subjected to analysis of variance (ANOVA) and separated with Fisher's protected least significant difference (LSD) test ($\alpha = 0.05$). For the biocontrol treatment comparisons, means were separated

Table 5. Total aflatoxin concentration in Aflasafe SN01-treated and untreated crops at harvest during 2014 and 2015 in four regions of The Gambia. Evaluation after 3-month storage was conducted only in 2014.

Year	Region ^w	Crop	Treatment	n	Total aflatoxin concentration (ppb)									
					At harvest					After storage				
					Min ^x	Max	Mean ^y	Variance	Reduction (%) ^z	Min	Max	Mean	Variance	Reduction (%)
2014	CRN	Maize	Treated	20	ND	28	2.6***	51	94.8	ND	5	0.7***	2	98.1
			Untreated	20	ND	314	48.9	5,351	–	ND	274	39.0	4,054	–
	CRN	Groundnut	Treated	14	ND	6	0.4	2	82.1	ND	43	2.9*	120	82.0
			Untreated	14	ND	19	2.3	25	–	ND	127	16.2	1,483	–
	NB	Groundnut	Treated	15	ND	23	2.7**	45	96.1	ND	6	1.2*	5	93.8
			Untreated	15	ND	553	68.6	20,705	–	ND	126	19.7	1,434	–
	WC	Groundnut	Treated	15	ND	36	4.4	113	97.5	ND	90	14.6	854	77.4
			Untreated	15	ND	1,727	172.9	231,432	–	ND	356	64.7	13,016	–
2015	CRN	Maize	Treated	8	ND	47	16.0*	251	79.8	–	–	–	–	–
			Untreated	8	10	227	79.3	7,605	–	–	–	–	–	–
	LR	Maize	Treated	12	ND	29	6.5***	103	83.3	–	–	–	–	–
			Untreated	12	4	168	39.0	2,512	–	–	–	–	–	–
	CRN	Groundnut	Treated	15	ND	ND	0.0*	0	100	–	–	–	–	–
			Untreated	15	ND	379	64.5	15,594	–	–	–	–	–	–
	NB	Groundnut	Treated	15	ND	ND	0.0*	0	100	–	–	–	–	–
			Untreated	15	ND	23	3.4	53	–	–	–	–	–	–
	WC	Groundnut	Treated	15	ND	ND	0.0	0	100	–	–	–	–	–
			Untreated	15	ND	97	9.9	757	–	–	–	–	–	–

^w CRN: Central River North, NB: North Bank, WC: West Coast, LR: Lower River.

^x ND: not detected. Aflatoxins were extracted as previously described (Agbetameh et al. 2018; Atehnkeng et al. 2008) and quantified with a scanning densitometer as previously described (Cotty and Cardwell 1999; Probst and Cotty 2012). Limit of detection = 1 ppb. For the purpose of the calculations, ND was considered 0 ppb.

^y Means of aflatoxin values were compared independently between treated and untreated samples in each region, in each year. Treated values with one, two, or three asterisks (*) significantly differed from their corresponding untreated treatment by Student's *t* test ($\alpha = 0.05$, 0.001, and 0.0001, respectively).

^z Percent reduction was calculated for each region in each year as follows: [(mean of untreated – mean of Aflasafe SN01-treated)/mean of untreated] × 100.

using paired Student *t* tests ($\alpha = 0.05$); in all cases, values of respective treated and untreated fields were compared. All statistical tests were conducted with SAS version 9.2 (SAS Institute Inc., Cary, NC).

Results

Aflatoxin concentration in groundnut sampled across The Gambia. Aflatoxin B and G varied among regions (Table 1). A total of 58

samples had no detectable aflatoxin content, and per region, these ranged from five (URN) to 14 (LR). WC had the highest ($P < 0.05$) average aflatoxins (195 ppb of B and 73 ppb of G) while LR had the lowest average aflatoxins (2 ppb of B and 0.5 ppb of G; Table 1).

***Aspergillus* section *Flavi* fungi in the groundnut samples.** A total of 1,608 *Aspergillus* section *Flavi* isolates were recovered from

Fig. 2. Percentage of groundnut and maize crops in each of four total aflatoxin concentration categories indicated by different colors. The outer circle shows Aflasafe SN01-treated crops while the inner circle shows untreated crops. In 2015, evaluations after storage were not conducted.

the groundnut. *A. flavus* L morphotype isolates produced smooth green-olive conidia and few, large sclerotia (>400 µm) (Cotty 1989). Isolates producing copious small sclerotia (<400 µm) and resembling the *A. flavus* S morphotype were classified as S morphotype-like fungi pending their status of aflatoxin-production ability (see below). *A. parasiticus* and *A. tamarii* were detected in some samples and were identified by their characteristic colony color and spore ornamentation (Klich and Pitt 1988).

The L morphotype dominated the communities in all regions (up to 85%, CRS), except in WC (42.9%), where S_{BG} strains had a frequency of 57.1% (Table 2). The frequency of S_{BG} strains ranged from 12.9 to 57.1%. *A. parasiticus* was detected only in samples from CRS (2.1%) while *A. tamarii* was detected in a single sample from CRN (Table 2). There were two isolates with S morphotype that produced only B aflatoxins, one isolate each in CRN and NB; those isolates were classified as S_B strains. Fungal densities varied per region ranging from less than 4,500 CFU/g to over 245,000 CFU/g (Table 2).

Aflatoxin production of the recovered *Aspergillus* fungi. The large majority (89.5%) of the recovered *Aspergillus* isolates (1,608 total) produced varying aflatoxin levels (Table 3). All *A. parasiticus* isolates produced both B and G aflatoxins, as expected. As mentioned above, all except two isolates with S morphotype produced both B and G aflatoxins (Table 2). The sole *A. tamarii* isolate recovered did not produce aflatoxins, as expected. Across the seven regions, 169 *A. flavus* L morphotype isolates did not produce aflatoxins. The range of atoxigenic isolates per region was 7 to 45 (Table 4).

Membership of atoxigenic fungi in AAVs composing the biocontrol product. None of the aflatoxin-producing L morphotype isolates belonged to the atoxigenic AAVs composing the biocontrol product. Among the 169 atoxigenic L morphotype fungi, 12 isolates (7.1% among the atoxigenic fungi) belonged to one of the four atoxigenic AAVs (Table 4). There was one member each of atoxigenic AAVs MS14-19 and M21-11, while there were five members each of atoxigenic AAVs SS19-14 and M2-7. AAV

Table 6. Population densities of *Aspergillus* section *Flavi* in soil, maize, and groundnut grain of Aflasafe SN01-treated and untreated fields during 2014 and 2015 in four regions of The Gambia

Region ^y	Crop	Treatment	CFU/g ^z			
			2014		2015	
			Soil before inoculation	Grain at harvest	Soil before inoculation	Grain at harvest
CRN	Maize	Treated	442	3,349,206	289	1,279,181
		Untreated	584	35,877,341	373	333,748
LR	Maize	Treated	—	—	172	408,953
		Untreated	—	—	168	47,318,086
CRN	Groundnut	Treated	1,980	53*	874	12,938
		Untreated	808	1,113	964	36,115
NB	Groundnut	Treated	281	776,368	343	5,139
		Untreated	270	134,475	234	14,025
WC	Groundnut	Treated	214	34,886	353	2,270
		Untreated	346	7,174,411	903	2,780

^y CRN: Central River North, LR: Lower River, NB: North Bank, WC: West Coast.

^z CFU/g: colony-forming units per gram of soil or grain. Values of treated soil before inoculation and grain at harvest with an asterisk significantly differed from those of the corresponding untreated treatments by Student's *t* test ($\alpha = 0.05$).

Table 7. Frequencies of *Aspergillus* section *Flavi* fungi in soil, maize, and groundnut grains of Aflasafe SN01-treated and untreated fields in four regions of The Gambia during 2014 and 2015

Year	Region ^y	Crop	Treatment	<i>Aspergillus</i> species distribution (%) ^z					
				Soil before inoculation			Grain at harvest		
				L	S _{BG}	T	L	S _{BG}	T
2014	CRN	Maize	Treated	95.0	0.0	5.0	100.0	0.0	0.0
			Untreated	100.0	0.0	0.0	100.0	0.0	0.0
	CRN	Groundnut	Treated	100.0	0.0	0.0	92.0	7.0	1.0
			Untreated	97.0	2.0	1.0	72.0	28.0	0.0
	NB	Groundnut	Treated	95.0	0.0	5.0	84.0*	16.0*	0.0
			Untreated	94.0	0.0	6.0	52.0	46.0	2.0
	WC	Groundnut	Treated	91.0	1.0	8.0	89.0*	11.0*	0.0
			Untreated	91.0	0.0	9.0	42.5	55.5	2.0
2015	CRN	Maize	Treated	100.0	0.0	0.0	100.0	0.0	0.0
			Untreated	98.0	2.0	0.0	100.0	0.0	0.0
	LR	Maize	Treated	97.0*	2.0	1.0	100.0	0.0	0.0
			Untreated	91.0	4.0	5.0	99.0	1.0	0.0
	CRN	Groundnut	Treated	100.0	0.0	0.0	89.0	11.0	0.0
			Untreated	86.5	12.5	1.0	87.0	13.0	0.0
	NB	Groundnut	Treated	99.0	0.0	1.0	72.0	28.0	0.0
			Untreated	89.0	8.0	3.0	69.0	31.0	0.0
	WC	Groundnut	Treated	96.0	0.0	4.0	83.0	17.0	0.0
			Untreated	98.0	2.0	0.0	52.0	48.0	0.0

^y CRN: Central River North, NB: North Bank, WC: West Coast, LR: Lower River.

^z L: *Aspergillus flavus* L morphotype; S_{BG}: fungi with S morphotype producing B and G aflatoxins; P: *A. parasiticus*; T: *A. tamarii*. Values of treated soil before inoculation and grain at harvest with an asterisk significantly differed from those of the corresponding untreated treatments by Student's *t* test ($\alpha = 0.05$).

SS19-14 occurred in two regions, while AAV M2-7 occurred in four regions (Table 4).

Quality test of the biocontrol product. All grains from each biocontrol product examined batch yielded 100% of carrier grains colonized by *A. flavus* and only fungi belonging to one of the four AAVs were detected (data not shown). None of the grains contained any other microorganism. Each atoxigenic AAV composed 25% $\pm$ 3% of the communities recovered from the carrier grains. The number of spores per g of biocontrol product was, on average, 3,500 $\pm$ 300.

Aflatoxin content at harvest and after storage from small-holder effectiveness trials. In 2014, at harvest, the average total aflatoxin was up to 4.4 ppb in treated crops (range = ND to 4.4 ppb; Table 5) while the average aflatoxin in untreated crops ranged from 2.3 to 172.9 ppb (Table 5). During that year, most untreated crops had unsafe, high aflatoxin levels with some samples containing >300 ppb and one with the hazardous content of 1,727 ppb. In 2015, treated maize had 6 and 16 ppb total aflatoxin in LR and CRN, respectively (Table 5). None of the treated groundnut during 2015 ($n = 45$) was contaminated with aflatoxins while it ranged from 3 to 64 ppb in untreated groundnut. In both years, there were 82 to 100% less aflatoxins in treated crops compared with untreated crops (Table 5).

In 2014, treated and untreated crops were tested for aflatoxin contamination 3 months after storage in farmer stores (Table 5). Treated crops accumulated significantly ($P < 0.05$) less aflatoxins (range = 0.7 to 14.6 ppb) than untreated crops (range = 16.2 to 64.7 ppb). Treated crops had 77.4 to 98.1% less aflatoxins than untreated crops after the storage period (Table 5).

At harvest, aflatoxin content variance in treated crops was 92 to 100% less than in untreated crops (Table 5). After storage (2014), aflatoxin content variance in treated crops was 89 to 99% less than in untreated crops (Table 5). Crops from biocontrol-treated fields contained higher proportions of samples with <4 ppb aflatoxin both at harvest and after storage (Fig. 2). In 2014, at harvest, the percentage of treated crops across regions with <4 ppb aflatoxin was 85% for maize and 87% for groundnut, while only 5% of maize and 7% of groundnut had >20 ppb aflatoxin. In 2015, treated groundnut in all regions contained undetectable aflatoxin levels, while 42% of maize in the examined regions had <4 ppb aflatoxin. After storage (2014), 95% of the treated maize and 82% of the treated groundnut had <4 ppb aflatoxin.

In contrast, untreated maize and groundnut at harvest in 2014 had 55% and 18%, respectively, of samples with >20 ppb aflatoxin (Fig. 2). After storage, 55% and 22% of untreated groundnut and maize samples, respectively, had >20 ppb aflatoxin. In 2015, 16 to 46% of untreated crops at harvest had >20 ppb aflatoxin (Fig. 2).

Fungal densities in field effectiveness trials. *Aspergillus* spp. densities in soil before biocontrol application were generally low in both treated (range = 172 to 1,980 CFU/g) and untreated fields (range = 168 to 964 CFU/g). In both years, fungal densities did not differ ($P > 0.05$) in any of the comparisons (Table 6).

There were many cases in which grains from untreated crops at harvest had higher fungal densities (range = 1,113 to 47.3 $\times 10^6$ CFU/g) than treated fields (range = 53 to 3.3 $\times 10^6$ CFU/g)

(Table 6). However, in both years, fungal densities did not differ ($P > 0.05$) in any of the comparisons, except for groundnut in CRN where higher densities occurred in untreated grains (Table 6). Values of fungal densities were always higher in grains at harvest than in soils before inoculation, regardless of treatment.

Distribution of *Aspergillus* section *Flavi* in field effectiveness trials. The *A. flavus* L morphotype dominated both soils before inoculation (range = 91 to 100%) and crops at harvest (range = 72 to 100%) during each year (Table 7). Although aflatoxin-producing ability was not evaluated, all fungi with S morphotype were considered to produce B and G aflatoxins and therefore were called S_{BG} strains; frequencies of S_B strains in the baseline study were low (only two isolates) and thus no efforts were made to characterize the aflatoxin production profile of fungi with S morphotype in this portion of the research. Before inoculation in 2014, S_{BG} strains were rarely detected and only isolated in untreated fields (range = 0 to 2%), *A. parasiticus* was not detected, while *A. tamarii* frequencies ranged from 0 to 9%. In 2014, S_{BG} strains were not detected in maize at harvest while in groundnut, S_{BG} strains ranged from 7 to 16% in treated crops and from 28 to 55% in untreated crops (Table 7).

In 2015, the occurrence of S_{BG} strains ranged from 0 to 2% in soils before application of biocontrol and from 2 to 13% in untreated plots. *A. tamarii* frequencies ranged from 0 to 5%. At harvest in 2015, S_{BG} strains were not detected in treated maize while in groundnut it ranged from 11 to 28% in the treated crops and from 13 to 48% in the untreated crops (Table 7). *A. parasiticus* was not detected in soils or grains of any given region, regardless of treatment.

Aflatoxin content in biocontrol effectiveness trials in commercially produced groundnut. In the three evaluated regions, the average aflatoxin concentration at harvest in biocontrol-treated groundnut ranged from 2.1 to 2.3 ppb and none exceeded 4 ppb (Table 8). Total aflatoxin in untreated crops at harvest on an average ranged from 12.8 to 53.1 ppb. Treated crops accumulated significantly ($P < 0.0001$) less aflatoxin (82 to 96% less) than untreated crops (Table 8). Aflatoxin variance in treated crops ranged from 0.1 to 0.4 while in untreated crops ranged from 92 to 1,846. Variance in treated crops was >99.5% lower than in untreated crops (Table 8).

Discussion

Human exposure to aflatoxins in The Gambia has been repeatedly quantified through urinary, blood, and breast milk analyses (Groopman et al. 1992; Wild et al. 1992; Zarba et al. 1992) and this exposure has been associated with growth faltering, immune suppression, and liver cancer (Kirk et al. 2006; Turner et al. 2003, 2007). However, very few studies have established aflatoxin levels in crops or foods in The Gambia. Two studies were conducted with very few samples and in another the origin of the samples is not clear (Hudson et al. 1992; Jallow et al. 2019; Xu et al. 2017). The current study is the first to establish a baseline for groundnut aflatoxin contamination across the country. In addition, the community structure of aflatoxin-producing fungi associated with groundnut was established and atoxigenic *A. flavus* native to The Gambia were identified for use in biocontrol products. Atoxigenic genotypes identified in the

Table 8. Total aflatoxin concentration in Aflasafe SN01-treated and untreated commercially produced groundnut at harvest during 2018 in three regions of The Gambia

Region ^x	Treatment	n	Total aflatoxin (ppb) ^y					Reduction (%) ^z
			Min	Max	Mean	Variance	95th percentile	
CRN	Treated	15	2	3	2.1***	0.1	3.0	96.0
	Untreated	15	11	170	53.1	1,846.0	135.0	
NB	Treated	15	1	3	2.3***	0.4	3.0	82.0
	Untreated	15	2	30	12.8	92.0	28.6	
WC	Treated	15	2	4	2.3***	0.4	3.3	93.6
	Untreated	15	12	71	35.9	292.0	63.3	

^x CRN: Central River North, NB: North Bank, WC: West Coast.

^y Total aflatoxins were quantified using Neogen Reveal Q+ for Aflatoxin kit and Neogene AccuScan Gold Reader. Limit of detection = 1 ppb. Treated values with three asterisks (***) significantly differed from their corresponding untreated treatment by Student's t-test ($\alpha = 0.0001$).

^z Percent reduction was calculated for each region as follows: [(mean of untreated – mean of Aflasafe SN01-treated)/mean of untreated] $\times$ 100.

current study are also in the biocontrol product Aflasafe SN01, originally isolated in Senegal, therefore allowing testing Aflasafe SN01 in The Gambia. Extensive field tests revealed that the biocontrol product is highly effective in The Gambia at decreasing aflatoxin content at harvest and during storage. Treated crops accumulated 82 to 100% less aflatoxins than untreated crops. This is the first report of the use and effectiveness of an atoxigenic biocontrol product in a nation other than the nation from which the active ingredients were originally isolated.

The baseline survey of aflatoxin concentrations revealed that contaminated groundnut is a major contributor to aflatoxin exposure of consumers in The Gambia and the resulting disease such exposure causes (Castelino et al. 2014; Kuniholm et al. 2008; Turner et al. 2000). Results showed that some groundnut contained dangerous aflatoxin concentrations, up to 1,400 ppb aflatoxin B₁ (Table 1), and was associated with extremely toxigenic fungi (Tables 2 and 3). Apart from health problems reducing the well-being and productivity of affected populations, aflatoxins pose a significant challenge to crop producers and exporters in West Africa, including The Gambia, aiming to reach domestic and international premium markets (Bandyopadhyay et al. 2016; Shephard 2008).

A recent study reporting aflatoxin levels in groundnut from The Gambia ($n = 1,168$) during an 11-year period (range = 0 to 2,581 ppb) found that 58% of the samples had aflatoxin below the 15 ppb Codex Alimentarius limit (Jallow et al. 2019). The study does not report the source of the samples (range per year = 13 to 276) and it is unknown if samples are representative of the whole country. We argue that the study that we conducted across The Gambia is the first aflatoxin baseline study for any crop/food in the country. Jallow et al. (2019) quantified aflatoxins using a methodology relying in spotting aflatoxin extracts until matching the fluorescence of the standards rather than directly quantifying with densitometry as reported in the current study. Such method could have underestimated aflatoxin levels in some samples.

Aflatoxin management strategies have been researched on over many decades for diverse crops (Aoun et al. 2020; Campbell et al. 2003; Hell et al. 2003; Lillehoj et al. 1980; Udomkun et al. 2017b; Waliyar et al. 2008, 2015). In the developed world, effective aflatoxin mitigation strategies are implemented, such as regulatory infrastructure, supplemental irrigation, harvesting advisories, processing, testing, and the burden of contamination is generally minor compared with the developing world. In developing and emerging nations, aflatoxin management strategies are either seldom used or not effective, the regulatory infrastructure is insufficient to remove most contaminated foods, and populations become continuously exposed to unsafe aflatoxin levels (Bandyopadhyay et al. 2016; Hanlon et al. 2019; Udomkun et al. 2017a). If available, aflatoxin control technologies are frequently used in isolation rendering crops vulnerable to aflatoxin contamination because use of single or few technologies do not provide adequate protection throughout the value chain.

In neighboring Senegal, use of an aflatoxin biocontrol product allows groundnut and maize to be produced with significantly less aflatoxins than untreated crops, both at harvest and after storage (Senghor et al. 2020). The government of The Gambia requested support from Plant Protection Service of Senegal, IITA, and USDA-ARS, to capitalize on experience to identify atoxigenic fungi with potential as biocontrol agents and to test a biocontrol product in maize and groundnut.

Aflasafe SN01, developed for use in Senegal (Senghor et al. 2020), is a biocontrol product registered with CSP/CILSS, the agency responsible for pesticide registration in 13 nations of the Sahel region, including The Gambia. Registration with CSP/CILSS allows product use in all 13 nations. However, it is general policy not to use biocontrol outside the region of normal distribution of its active ingredients in order to ensure superior performance in the target agroecosystems and to avoid any concerns about negative effects of introducing exotic genotypes. To meet these concerns, the AAVs in Aflasafe SN01 were sought in crops produced in The Gambia. Among 169 atoxigenic isolates cultured, 12 were found to be one of the four active ingredients of the biocontrol product Aflasafe SN01, and these

occurred in five of the seven regions (Table 4). Upon identification of the four active ingredients of Aflasafe SN01 in The Gambia, it was decided to avoid the time-consuming, resource-intensive process of identifying new active ingredients and to move directly into testing the product across The Gambia. The biocontrol product was tested during two cropping seasons in both groundnut and maize and was found to be highly efficient in limiting aflatoxin content by displacing aflatoxin producers (Tables 5, 6, and 7; Fig. 2). The product was also used by commercial farmers who reported excellent effectiveness with greater than 80% reductions in aflatoxin content and no more than 4 ppb in any lot (Table 8). Overall, the current study supports biocontrol as an aflatoxin management tool that groundnut and maize growers in The Gambia can exploit to limit crop aflatoxin contamination, both at harvest and after storage.

The biocontrol product protected maize and groundnut throughout all critical phases of contamination with significantly reduced aflatoxin contamination both at harvest and after storage (Table 5). Furthermore, variance in aflatoxin content was always several-fold lower in treated crops compared with untreated (Table 5). Reduced variance in treated crops means less risk of aflatoxin contamination (Senghor et al. 2020). Biocontrol with atoxigenic fungi does not always result in total elimination of aflatoxins in treated crops (Bandyopadhyay et al. 2019; Senghor et al. 2020). However, in the current study, no aflatoxins were detected in any of the treated groundnut produced during 2015 (Table 5; Fig. 2). The observed highly significant reductions during the effectiveness trials (2014 and 2015) occurred in the absence of improved agronomic practices such as weeding fields on time, timely harvesting, drying groundnut in small heaps with pods avoiding contact with the soil (Waliyar et al. 2008), which may be expected to provide additional benefits (Bandyopadhyay et al. 2019). Aflatoxin levels in some crops were higher at harvest than after storage, for both treated and untreated crops, but there were no consistent trends (Table 5). However, there were no significant differences ($P > 0.05$; Student's *t*-test) when either treated or untreated crops were compared at harvest and after storage. For example, on an average, aflatoxin levels in maize in CRN were 48.9 ppb at harvest and 39.0 ppb after storage, but these were not significantly different.

Regardless of treatment, farmers sorted the grains before storage, as per their practices. On one hand, sorting contributed to less aflatoxins after storage in untreated crops from three of the four comparisons. On the other hand, on an average, reductions resulting from sorting of untreated crops were in most cases insufficient to make aflatoxin content safe for consumption after the storage period (Table 5). Although sorting is an effective practice to remove mycotoxin contaminated grains (Aoun et al. 2020; Matumba et al. 2015), it does not always result in reducing aflatoxin content to acceptable levels and/or may concentrate aflatoxins in rejected grains that ultimately are consumed (Matumba et al. 2015). In The Gambia, groundnut purchased from markets were reported to contain substantially less aflatoxin when moldy kernels were separated (Xu et al. 2017). In that study, a group of women, who traditionally sort the groundnut using different techniques, were trained to conduct the sorting process. Women participating in the current study were not given any training and their sorting practices were not monitored. The increased aflatoxin content in both treated and untreated groundnut in CRN and in treated groundnut in WC (Table 5) could be the result of improper sorting, more deficient storage practices than in other areas, high moisture content prior to storage, or a combination of those factors. Regardless, as stated before, none of the individual at harvest versus after storage comparisons were significantly different.

There is the notion that biocontrol treatment does not provide adequate postharvest protection (e.g., aflatoxin below 10 ppb) (Ehrlich et al. 2015; Gressel and Polturak 2018; Villers 2014), although postharvest benefits have been reported in the U.S. (Dorner and Cole 2002). Recently, that hypothesis was tested by subjecting maize from treated fields in the U.S. to a lab storage assay with uninterrupted aflatoxin formation conducive conditions (6 days, 28°C). The experiment revealed that aflatoxin increased

to unsafe levels (Kinyungu et al. 2019). In contrast, in our studies, the average aflatoxin content in sorted biocontrol-treated grains after a 3-month storage period using traditional practices resulted in safe aflatoxin levels in three regions (average range = 0.7 to 2.9 ppb), while in the fourth region, the average concentration in treated, stored groundnut was 14.6 ppb. In contrast, aflatoxin in similarly sorted, stored untreated grains ranged from 16.2 to 64.7 ppb (Table 5). Treated crops had considerably more (up to 100%, depending on the crop and year) samples with <4 ppb aflatoxin while untreated crops sometimes had more than 50% of the samples with >20 ppb (Fig. 2). Use of biocontrol in Senegal over 5 years also resulted in treated, sorted grains ($n = 268$) containing on average less than 10 ppb (range = 0.5 to 9.9 ppb) after a 4-month storage period using traditional practices while average aflatoxin for sorted, stored, untreated grain ($n = 268$) ranged from 10.1 to 64.9 ppb (Senghor et al. 2020). Evaluation of postharvest benefits under artificial systems may not reliably estimate benefits of biocontrol in limiting aflatoxin content to below tolerance thresholds during real-life storage conditions. When simulating poor storage conditions, treated grains accumulate less aflatoxins than untreated grains, although experiments under artificial conditions sometimes yield stored treated crops with reduced but still unsafe aflatoxin levels (Atehnkeng et al. 2014; Bandyopadhyay et al. 2019). Ultimately, the level of postharvest protection provided by atoxigenic biocontrol agents will depend on factors such as climatic conditions, sorting practices, storage conditions, period of storage, insect control, and fungal load, among others.

Regardless of the biocontrol postharvest benefits detected in real-life studies conducted in Senegal and The Gambia, an integrated set of good preharvest and postharvest practices is included in the aflatoxin management system that IITA and partners promote in The Gambia and other nations. A system including good agronomic practices, biocontrol, sorting, drying, and improved storage and processing practices, along with policy and institutional solutions, will result in decrease aflatoxin content throughout the value chain.

Deliberate application of biocontrol organisms has raised concerns of the possibility of i) causing ecological imbalances affecting populations of microorganisms not targeted (Köhl et al. 2019) and, in the case of atoxigenic biocontrol products, ii) increased quantities of *Aspergillus* spp. throughout the environment that could increase kernel rot (Bhandari et al. 2020). However, the amount of *Aspergillus* spores in treated and untreated crops is dependent on environmental conditions and available resources for fungal reproduction and not primarily driven by biocontrol application (Bandyopadhyay et al. 2016). This was evident in 2014, where untreated maize in CRN had on an average 10 times more CFU/g than treated maize (Table 6). On the other hand, in the following year in CRN, there was a threefold fungal increase in treated compared with untreated maize although the densities (1.3×10^6 CFU/g) were lower than in untreated maize during the previous year (35.9×10^6 CFU/g, Table 6). In 2014, groundnut had over 20 times more CFU/g in untreated crops in two regions (Table 6). There were a few cases in which higher, but statistically similar fungal densities were detected in treated crops (Table 6).

The possibility of decreased fungal densities as a result of biocontrol treatment has not been discussed because a priori intuition suggests that biocontrol application will automatically increase the densities of *Aspergillus* spp. in the environment. On the other hand, in the absence of biocontrol application, densities of *Aspergillus* fungi can increase rapidly as a result of the exploitation of crop-associated nutrients and explosive, rapid reproduction driven by founder events of single or few *A. flavus* VCGs (Ortega-Beltran et al. 2020). Rapid reproduction and dispersal of few *A. flavus* VCGs have been detected in untreated areas over diverse, highly contrasting environments in Mexico (Ortega-Beltran and Cotty 2018). Also, in the U.S., subpopulations containing aflatoxin-producers and non-toxin producers were found across a vast area (Drott et al. 2019), which may have been caused by founder events as well. A rapid increase of few genotypes generated by founder events followed by intense clonal reproduction and efficient dispersal (Islam et al. 2018) may have occurred across some of the examined regions in The

Gambia and therefore higher fungal densities were detected in untreated crops (e.g., maize in CRN in 2014, Table 6). Research to determine VCG community structures in untreated soils and crops of the current study is needed to elucidate the complexity of genotypes responsible for the large population increases in untreated fields.

Biocontrol application appears, in crops from some regions, to have prevented the rapid increase of one or a few genotypes occurring in neighboring, untreated fields. One of the principles of the biocontrol technology is that the timely introduction of biocontrol genotypes restricts the reproduction of populations with greater aflatoxin-producing potential through competitive exclusion (Cotty 2006; Mehl et al. 2012). Competitive exclusion thus prevents natural increases of highly toxigenic *Aspergillus* spp. communities, regardless of the magnitude of increase supported by the local environment and resources. There will be some areas or years where large increases in nearby untreated crops will not occur and therefore either equal or greater fungal densities will be detected in treated crops compared with untreated crops. Biocontrol treatment in Senegal over 5 years did result in higher ($P < 0.05$) fungal densities in treated crops of some areas, in certain years (Senghor et al. 2020).

The proportion of *Aspergillus* species in soils before treatment across areas and years were generally similar in treated and untreated fields across regions, crops, and years (Table 7). Both treated and untreated soils where maize was evaluated contained low frequencies of S_{BG} strains and in grains at harvest, maize was dominated by the L morphotype, with S_{BG} strains only detected in a few fields of LR in 2015 (Table 7). Thus, influences of treatments on S_{BG} strain incidence in maize could not be evaluated. On the other hand, there were significant differences in proportions of both the L morphotype and S_{BG} strains in treated and untreated groundnut. Proportions of S_{BG} strains were consistently lower in treated groundnut, particularly when S_{BG} strains were high in untreated crops (Table 7). In the current study, low frequencies of the highly toxigenic species *A. parasiticus* were detected (Tables 2 and 7). This species is infrequently associated with groundnut cropped in The Gambia. Similar poor association of *A. parasiticus* with groundnut has been reported in Senegal and Ghana (Agbetiameh et al. 2018, 2020; Senghor et al. 2020). In other groundnut-producing regions, *A. parasiticus* is a more important member of the aflatoxin-producing communities (Horn 2003; Kachapulula et al. 2017b; Klich 2007).

After the field effectiveness stage and registration, the biocontrol product has been used commercially. In the 2018 cropping season, commercial farmers belonging to the National Food Security Processing and Marketing Corporation (NFSPMC) of The Gambia treated 5,000 ha of groundnut as a part of an integrated aflatoxin management strategy mentioned earlier. Performance of the biocontrol was monitored at harvest in groundnut from 45 treated fields and paired untreated fields. All treated groundnut had sufficient quality to be commercialized in the most stringent markets (<4 ppb total aflatoxin), while a large proportion of untreated groundnut failed to reach safe aflatoxin levels (Table 8). Indeed, part of the biocontrol-treated groundnut was sold into European markets, which is very difficult to achieve for SSA farmers due to rigid aflatoxin regulations (Ramsay et al. 2019). The aflatoxin testing was performed in-situ, contrary to the predominant practice of doing so in laboratories. The in-situ testing provides farmers, extension officers, buyers, and agro-dealers the opportunity to participate in the process and witness the results. Demonstrating to farmers that their crops contain safe or unsafe aflatoxin levels increases the potential for adoption of management strategies. It is difficult to convince farmers of the importance of aflatoxin management when testing is conducted in a far-away laboratory. The in-situ testing has increased confidence in biocontrol as part of an aflatoxin management system. It most importantly allows farmers to rapidly know if they can sell their crops in premium markets to obtain higher income. Following segregation of groundnut lots with low aflatoxin content as revealed by the in-situ testing, some aflatoxin-conscious buyers and farmers submitted samples to an approved laboratory. Those buyers and farmers randomly collected 25

to 70 kg of kernels, milled them using a hammer mill, and thereafter submitted a homogenized 25 g subsample for analyses.

We have argued that use of native atoxigenic genotypes as active ingredient fungi of biocontrol formulations provides better chances to reduce crop aflatoxin content compared with the use of exotic fungi (Agbetiameh et al. 2019, 2020; Bandyopadhyay et al. 2016; Ortega-Beltran and Bandyopadhyay 2019; Senghor et al. 2020). Native fungi are locally adapted to environmental conditions and have higher chances to outcompete fungi residing in treated fields (Mehl et al. 2012; Probst et al. 2011). Therefore, using exotic atoxigenic fungi in biocontrol programs may not result in reductions to aflatoxin-compliant levels because of poor active ingredient adaptation to the target region. Besides, the use of atoxigenic exotic fungi may not be accepted by biopesticide regulatory authorities in most countries due to the perceived risk of environmental impact. We continue supporting the principle that use of atoxigenic fungi as biocontrol agents should be pursued only when there is evidence that these fungi are native to a target area in order to increase aflatoxin reductions and to alleviate any concerns related to the introduction of exotic organisms. There are many atoxigenic germplasm in all regions where aflatoxin-producing fungi thrive (Mehl et al. 2012).

Here we document for the first time the use in two countries of the same aflatoxin biocontrol product, Aflasafe SN01, composed of active ingredient fungi native to both nations, Senegal and The Gambia. Manufacturing and distribution responsibilities of Aflasafe SN01 have been licensed to BAMAARE SA, a private company in Senegal (Schreurs et al. 2019; Senghor et al. 2020). BAMAARE's manufacturing facility, now operational in Kaolack, Senegal, produced the biocontrol product for use during the 2019 and 2020 cropping seasons in both Senegal and The Gambia. The current results suggest that large-scale use of Aflasafe SN01 as a centerpiece of an integrated aflatoxin management system will provide substantial benefits to human health, food security, income, and trade sectors in multiple nations in West Africa, which suffer from recurrent aflatoxin contamination events.

Acknowledgments

Special thanks to the maize and groundnut farmers in The Gambia for willingly allowing the trials to be conducted in their fields and participating in the current study. We thank Antony Carvalho and Mustapha Coley from NFSPMC, for supporting the field trials.

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