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Aspergillus flavus diversity on crops and in the environment can be exploited to reduce aflatoxin exposure and improve health

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Humans and animals are exposed to aflatoxins, toxic carcinogenic fungal metabolites, through consumption of contaminated food and feed. *Aspergillus flavus*, the primary causal agent of crop aflatoxin contamination, is composed of phenotypically and genotypically diverse vegetative compatibility groups (VCGs). Molecular data suggest that VCGs largely behave as clones with certain VCGs exhibiting niche preference. VCGs vary in aflatoxin-producing ability, ranging from highly aflatoxigenic to atoxigenic. The prevalence of individual VCGs is dictated by competition during growth and reproduction under variable biotic and abiotic conditions. Agronomic practices influence structures and average aflatoxin-producing potentials of *A. flavus* populations and, as a result, incidences and severities of crop contamination. Application of atoxigenic strains has successfully reduced crop aflatoxin contamination across large areas in the United States. This strategy uses components of the endemic diversity to alter structures of *A. flavus* populations and improve safety of food, feed, and the overall environment.

Keywords: *Aspergillus flavus*; aflatoxin; food safety; biocontrol; competitive exclusion

Introduction

The filamentous fungus *Aspergillus flavus* is the primary causal agent of food and feed contamination with the severely toxic fungal metabolites, aflatoxins.[1–4] A wide variety of food crops including maize, cottonseed, peanuts, and tree nuts are susceptible to infection and subsequent aflatoxin contamination.[1] The most common aflatoxin, aflatoxin B_1, is a toxic fungal metabolite known to be carcinogenic and teratogenic for both humans and animals.[5,6] It is the only mycotoxin classified as a group 1a human carcinogen by the International Agency for Research on Cancer.[7] Acute health effects of aflatoxin exposure from consumption of highly contaminated food include liver cirrhosis and death.[8] Chronic consumption of sublethal concentrations is associated with liver cancer, growth impairment, and immune suppression.[9–11] In most developed nations, aflatoxins within food and feed are limited by strictly enforced regulations that result in significant economic loss for producers and processors of contaminated crops.[12,13] In developing nations, less effective enforcement of regulations results in chronic exposure to aflatoxins and perennial deleterious effects on human health. The most severe episodes of acute aflatoxicosis with numerous deaths occurred over the past decade.[8,9,14]

Communities of aflatoxin-producing fungi resident in agricultural and native ecosystems are complex assemblages of genotypically and phenotypically diverse individuals.[1] Average aflatoxin-producing potential of *A. flavus* populations is an important determinant of the incidence and severity of aflatoxin contamination events.[3,15,16] In warm regions of the United States, growers of susceptible crops have experienced repeated and severe losses from aflatoxin contamination.[12] For many of these

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growers, a form of biological control in which native atoxigenic (i.e., do not produce aflatoxins) isolates of *A. flavus* are used to competitively exclude aflatoxin producers is the only viable solution for mitigating contamination.[17–21] Atoxigenic strain applications can be made without increasing the proportion of the crop infected by *A. flavus* and without increasing the overall quantity of *A. flavus* on the crop and in the environment.[22,23] Atoxigenics are naturally associated with crops and, even in the absence of their application, they displace toxigenic *A. flavus* and reduce contamination.[24] Application of native atoxigenic biocontrol strains simply increases the frequency of this natural phenomenon.[17,23] Atoxigenics typically provide over 80% displacement of aflatoxin producers and an associated 80% reduction in contamination with a single application of 10 kg/ha of formulated product (Fig. 1).[18,25,26]

Two atoxigenic strains are currently registered as biopesticides by the EPA,[20] but diverse atoxigenic strains are being developed for biocontrol across the globe. Individuals within *A. flavus* populations vary in aflatoxin-producing potential, virulence, host specialization, competitive ability, and other potentially adaptive traits. Thus, diversity within *A. flavus* populations can be exploited to develop and improve strategies for reducing the average aflatoxin-producing potential of *A. flavus* communities, thereby reducing human exposure to aflatoxins both through consumption of contaminated food and through inhalation of fungal spores and crop fragments.[27–30] This review summarizes current knowledge on diversity and adaptation of *A. flavus* and how human activities, including deliberate modification of *A. flavus* communities as part of aflatoxin management strategies, impact *A. flavus* population structure and the resulting exposure of humans to aflatoxins. Furthermore, future directions for development and improvement of aflatoxin mitigation measures, specifically in the context of biocontrol with atoxigenic strains, are discussed.

Biology of *Aspergillus flavus*

A. flavus is ubiquitous in the environment and proliferates both as a saprophyte[31] and an opportunistic pathogen of plants and animals[32] including humans.[33,34] It is most prevalent in warm regions, especially between latitudes 35 N and 35 S.[1] In addition to influencing human health through exposure to aflatoxins, *A. flavus* is second only to *A. fumigatus* in nosocomial aspergillosis and is the dominant causal agent among the aspergilli of sinusitis and fungal keratitis.[34] Though primarily studied in agroecosystems, *A. flavus* is common in a variety of natural habitats including the air, soil, and plants of the Sonoran Desert;[22,35] and freshwater, marine,[36] and indoor environments.[34]

The saprophytic phase of the *A. flavus* life cycle occurs primarily in soil where the fungus colonizes organic debris and resides as either mycelia or heavily melanized survival structures called sclerotia.[37,38] When environmental conditions are favorable (e.g., elevated temperature), propagules within debris give rise to conidiophores harboring airborne conidia that are subsequently dispersed throughout the environment.[39] Under appropriate conditions, wind and insect dispersal of conidia to plants results in colonization, infection, and, within susceptible hosts, production of aflatoxins. Conidia produced on plant surfaces serve as inoculum for secondary infections, several cycles of which may occur in a single growing season.[22,40] Infected plant and other organic debris within and on soils serve as reservoirs of *A. flavus* for subsequent dispersal to susceptible hosts and/or nonliving food sources.[41]

The success of genotypically and phenotypically diverse *A. flavus* at each stage in the disease/life-cycle and in different niches affects the structure of aflatoxigenic fungal communities in the environment. Currently, aflatoxin management through application of atoxigenic biocontrol strains aims to displace toxigenic *A. flavus* on a target crop before and during the initial phase of infection.[18] Displacement of toxigenic fungi from a variety of niches rather than just the target crop, and during multiple stages of fungal life cycles including the saprophytic phase, may be necessary to achieve optimal long-term reductions in aflatoxins and other mycotoxins of health and economic concern (e.g., those caused by *Fusarium* species). The genetic and phenotypic diversity of *A. flavus* in various niches provides germplasm of potential value in biocontrol that can be exploited to reduce the prevalence of aflatoxigenic fungi in the environment.

Phenotypic and genotypic diversity in *A. flavus*

Phenotypes of *A. flavus* vary widely, and the species can be divided into two major morphotypes known

Figure 1. Competitive exclusion of aflatoxin producers with an atoxigenic biocontrol strain of *A. flavus* is currently the most effective means for growers to reduce aflatoxin contamination in susceptible crops. Contamination of maize (A) and cottonseed (B) greatly reduces market value, but biocontrol has proved to be an effective management strategy in these crops. Large-scale manufacturing (C, D) and commercial application of biocontrol strain AF36 (E) has been achieved for cotton, maize, and pistachio through a partnership between the Arizona Cotton Research and Protection Council (ACRPC) and the Agricultural Research Service of the U.S. Department of Agriculture (USDA-ARS).[18] Grain coated with the fungus is applied to fields before emergence of susceptible crop components (E). The biocontrol product as applied (left) and following fungal growth (right) are shown in panel (D).

as the L and S strains. S strain isolates produce numerous small (average diameter <400 μm) sclerotia and relatively few conidia, while L strain isolates produce fewer large (average diameter >400 μm) sclerotia and relatively large quantities of conidia.[42] Aflatoxin-producing potential varies widely within *A. flavus* ranging from no aflatoxin production (atoxigenic) to production of over 10^6 ppb in susceptible crop components.[1,29,43] L strain isolates of *A. flavus* produce on average lower

levels of aflatoxins than S strain isolates[3,42] and atoxigenic L strain isolates have been reported from many regions.[4,15,44,45] In contrast, S strain isolates of *A. flavus* produce relatively high levels of aflatoxins, and reports of atoxigenic S strain isolates are lacking.[42] There are other species of aflatoxin-producing fungi with S strain morphology, and these also produce large quantities of aflatoxins.[16] Aflatoxin-producing potential is not associated with either virulence[30,42] or competitive ability during crop infection.[30] The genetic basis for atoxigenicity varies among isolates and may include both single polymorphisms that disrupt production of key enzymes[46] and large deletions in the aflatoxin biosynthesis gene cluster.[47–49]

Determining the primary etiologic agent(s) (i.e., causal agents) of aflatoxin contamination is critical for predicting risk of contamination events and for designing and implementing management strategies. However, the most prevalent type of *A. flavus* infecting a crop is not necessarily the most important etiologic agent of contamination. For example, even when the S strain causes a small proportion (5 or 10%) of the total *A. flavus* infections, it can be the most important causative agent of a contamination event since the S strain produces very high aflatoxin concentrations.[3,14,16,24,50] Thus, aflatoxin management strategies that reduce frequencies of the S strain morphotype may be particularly effective at reducing contamination.[51]

Great genetic diversity among *A. flavus* isolates can be resolved from molecular characteristics including chromosomal karyotypes,[52] mitochondrial, and nuclear restriction fragment length polymorphisms (RFLPs),[53–56] microsatellites,[57,58] nucleotide sequence data,[59,60] and the presence or absence of particular genes and/or indels.[16,47,61] A vegetative incompatibility system mediates self/nonself recognition among genotypically diverse *A. flavus* individuals, and membership of isolates within vegetative compatibility groups (VCGs) provides additional criteria to assess genetic diversity within *A. flavus* populations. Individuals that undergo anastomosis must possess identical alleles at the loci governing vegetative compatibility in order to form a stable heterokaryon and allow gene flow between those individuals. In contrast, gene flow is restricted between dissimilar individuals.[62,63] Phenotypic characteristics, including sclerotial morphology and ability to produce aflatoxins, are typically conserved within a VCG,[64–67] and thus VCGs are commonly treated as functional genetic/ecological/epidemiological units.

Vegetative compatibility analyses provide insight into population genetic diversity as well as changes in compositions of crop-associated *A. flavus* associated with various events.[54,67–71] *A. flavus* populations are composed of many VCGs, and multiple VCGs may occur within a single crop component or aliquot of soil.[70] Some VCGs are common in the environment whereas others are rarely isolated,[67,70,71] and relative frequencies of morphotypes and VCGs vary among crops, fields, regions, seasons, and years.[15,22,44,67,72–75] Thus, each agroecosystem has its own unique, continuously fluctuating assemblage of genetically diverse *A. flavus* that must be managed to minimize crop contamination. Selection of agroecosystem-adapted atoxigenic strains for biocontrol should therefore include region-wide analyses of the genetic and phenotypic diversity within *A. flavus* populations.

DNA sequence data confirm that isolates within a VCG are closely related and genetically distinct from other VCGs. For example, with sequence data from three loci, 36 L strain isolates from six VCGs were divided into four lineages.[59] RFLPs provide greater resolution and were able to separate 75 isolates from 44 VCGs into distinct VCG-defining lineages with sufficient resolution to detect variation among isolates within a VCG.[54] Array comparative genomic hybridization (aCGH) indicated nearly identical gene content within a VCG but up to 2% differences in which genes are present between VCGs.[76] A population study using 24 microsatellite (or simple sequence repeat (SSR)) markers to examine 243 isolates from three VCGs found genetic variation among and within VCGs, but each of the three VCGs were highly resolved, and genetic exchange among them was not detected.[58] Isolates were from sympatric populations, diverse geographic origins, and multiple years, indicating VCGs are genetic lineages that can be widespread in the environment and persist over time. Analysis of variability in microsatellite loci within and among the three VCGs allowed estimates of the time of divergence between the VCGs of 10,000 to 60,000 years before present. Thus, divergence predates the advent of agriculture.[58] Following divergence, VCGs have, at least in some cases, evolved clonally, sometimes in association with agroecosystems. The potential for a sexual

stage has been shown in laboratory crosses,[77,78] but the importance of sexual reproduction in shaping *A. flavus* populations in nature and in the process of *A. flavus* evolution and adaptation to agroecosystems is unclear.

Geiser *et al*. raised sexual recombination as a potential stumbling block to the use of atoxigenic isolates as biocontrol agents, presumably due to the creation of highly competitive toxigenic recombinants.[60] In our work, exotic atoxigenic strains have never been introduced to a region; only atoxigenic strains native to target agroecosystems are used. Indeed the most widely distributed atoxigenics are preferred as distribution is used as a proxy for success within the environment. As such, these VCGs have coexisted with the aflatoxins producers they are displacing for over 10,000 years, and no new opportunity for recombination is created by atoxigenic strain use. While sexual reproduction has been reported in the lab under stringent conditions,[77] including between the two biocontrol strains currently approved for use in the United States and other *A. flavus* isolates,[78] genetic data from natural populations provide no evidence for genetic exchange between VCGs.[58] Sexual reproduction, if it occurs in natural populations, is apparently at a low frequency. Even under highly favorable conditions, low fertility[79] and required long time frames[77] observed in laboratory crosses suggest rare recombination. Sexual reproduction may contribute to the evolution of new genotypes within *A. flavus*, but current population genetic studies suggest the importance of sexuality lies on an evolutionary time scale, not an epidemiological one.[58] However, if recombination did occur between an atoxigenic genotype and an aflatoxins producer, the result would be an increased diversity of atoxigenic haplotypes.

Adaptation in *A. flavus*

The broad spectrum of degrading enzymes produced by *A. flavus* isolates as well as their ability to obtain nutrition both pathogenically and saprophytically from a wide variety of hosts and substrates suggests a lack of specialization by the species.[32,80,81] However, variability among isolates in production of pectinases, hydrolytic enzymes involved in maceration of plant host tissues, suggests differential adaptation to plant hosts.[80,82] Ability of isolates to spread between and rot cotton locules is correlated with the production of a specific pectinase P2c,[83,84] and P2c knock-out mutants have reduced virulence to cotton.[85] Variable pectinase production among *A. flavus* isolates suggests some isolates are more adapted to plants whereas others are more adapted to niches where ability to macerate plant tissues provides less of an advantage.[82]

Phenotypic variation among *A. flavus* isolates provides further evidence for niche differentiation. For example, production of large quantities of sclerotia and aflatoxins may confer an adaptive advantage in soils where long-term survival and defense against insect grazing may be essential for success. In some studies, soil populations of *A. flavus* produced, on average, more aflatoxins and sclerotia than *A. flavus* from crops.[86,87] Production of high levels of aflatoxins and sclerotia and a lack of pectinase P2c production by about 50% of S strain isolates from Arizona suggest at least some members of this morphotype are more adapted to soil than to crop environments.[42,82] Aflatoxin producers with S morphology have been identified as the primary etiologic agents of several contamination events.[14,50] If these isolates are best adapted to soil environments, they may be particularly vulnerable to competitive displacement by atoxigenics in the crop.[4,24] Conversely, in soils during fallow periods, S strain may be favored over crop-adapted atoxigenics, and therefore applied atoxigenic strains may have comparatively less persistence in the environment. The identity and frequency of VCGs differ between soil and crop populations even within a single field, and some VCGs common in soil are not easily detected on the crop and vice versa.[70,87] Such soil resident *A. flavus* genotypes may be overwintering between preferred hosts (either animal or plant) or may be better adapted as saprophytes than as pathogens. Regardless of that, competition between soil-adapted *A. flavus* and applied atoxigenic biocontrol strains during periods of overwintering probably influences the persistence of atoxigenics in the environment. Thus, utilization of both soil- and crop-adapted atoxigenics may be necessary to achieve optimal long-term modification of the aflatoxin-producing fungal community and to both increase additive benefits and reduce the necessity of yearly applications.

Ability of genotypes to compete during host infection may have a greater influence on the *A. flavus* population structure than virulence. This is a type of host specialization in which a genotype has

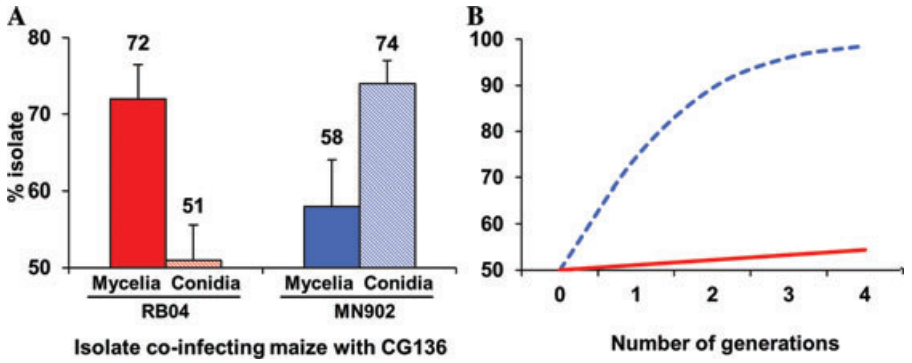

Figure 2. Differential ability of *A. flavus* isolates to compete during maize kernel infection and sporulation (A) and the predicted influence of these competitive differences on *A. flavus* population dynamics over time (B). Isolates RB04 and MN902 were each co-inoculated in equal proportions on maize kernels with an isolate from a common toxigenic VCG, CG136. Isolate percentages (A) from kernel-infecting mycelia and conidia produced during infection were determined by quantifying isolate-specific single nucleotide polymorphisms from total *A. flavus* mycelia and conidia DNA with pyrosequencing. RB04 outcompeted CG136 during maize kernel infection but not during sporulation, whereas MN902 was significantly more competitive during sporulation than during co-infection of kernels with CG136. The predicted influence of competitive differences detected after one cycle of infection and reproduction (A) on proportions of RB04 (solid line) and MN902 (dashed line) within the *A. flavus* population over time are shown in panel B. Conidia produced during host infection contribute to secondary infections, many of which may occur in a single growing season, and presumably shift population structure with each subsequent cycle of infection and reproduction. Calculations are based on the assumption that the isolates initially encounter the crop in equal proportions (50% each) and that the *A. flavus* "population" comprises only CG136 and either RB04 or MN902. And although RB04 comprised a greater percentage of the total *A. flavus* infecting the crop (relative to CG136) after one cycle of infection and reproduction, the superior ability of MN902 to compete during sporulation may contribute to its success on the crop over time. Data are derived from Ref. 30.

adaptations that confer advantage during colonization and infection of some hosts but not necessarily others.[30,88] Indeed, differential competitive ability among isolates on plant hosts indicates crops can select for certain genotypes within *A. flavus* populations through influences on outcomes of competition (Fig. 2). The notion that hosts influence the *A. flavus* population structure is supported by studies in which both aflatoxin-producing potential of *A. flavus* populations[89,90] and quantities and frequencies of morphological and genetic types[73,74,91] were found to vary among crop hosts. Furthermore, though lineages are not exclusively associated with a particular host, certain genotypes of *A. flavus* are more likely to be associated with specific hosts or habitats than others[36] suggesting divergence in host specialization within *A. flavus* populations.

During competition for plant host substrates, some genotypes of *A. flavus* are highly successful during host invasion and tissue ramification whereas others compete poorly during capture of substrates but are highly competitive during sporulation.[30] These differential strategies in response to competition likely have important impacts on the *A. flavus* population structure and the epidemiology of *A. flavus* infection and aflatoxin contamination. *A. flavus* individuals highly competitive during capture of host substrates have the greatest influence on aflatoxin content within infected host tissues.[30,42] In contrast, an isolate highly competitive during sporulation may come to dominate the population during multiple cycles of reproduction[22,40] even if it is a relatively poor competitor during crop infection[30] (Fig. 2). However, when environmental conditions are not conducive to sporulation, dispersal, and secondary infection, genotypes dominant within host tissues are more adapted to long-term survival in cropping systems.[41] Rather than one strategy being superior over the other, differential behavior of isolates during competition may reflect niche partitioning that allows for maintenance of multiple genotypes within *A. flavus* populations over time and space.[92] Diverse adaptive strategies to various hosts presumably influence ability of atoxigenics to infect and multiply on crops and to persist in the environment. Competitive success during all stages of the *A. flavus* disease/life cycle and on multiple hosts and nonliving substrates are important criteria for the selection of atoxigenic isolates for potential use in aflatoxin mitigation.

Impact of human activity on *A. flavus* populations

Crop rotations influence aflatoxin contamination by altering both density and structure of *A. flavus* populations. For example, colony-forming units (CFU) of *A. flavus* increased in soils continuously rotated from maize to groundnut,[93] and, likewise, CFU of *A. flavus* were greater in residues from plots cropped continuously to maize than from plots cropped with a soybean–maize rotation.[94] In southern Texas, significantly higher CFU of *A. flavus* occurred in soils previously cropped to maize than those previously cropped to cotton or sorghum.[74,91] Higher population densities of *A. flavus*, however, do not always translate to higher crop contamination. Aflatoxin contamination is influenced by both density and structure of *A. flavus* populations, and crops that favor high S strain incidences increase the aflatoxin-producing potential of the *A. flavus* population.[15] In southern Texas, soils previously cropped to cotton and sorghum had higher frequencies of the S strain than those cropped to maize (Fig. 3).[74,91] Crop rotations may be manipulated to lower incidences of the highly toxigenic S strain and to favor success of applied atoxigenic strains. This is just one potential benefit that may arise from extended research on the influence of crop rotations on the *A. flavus* population structure.

Climate influences the density and structure of *A. flavus* populations as well as the extent to which fungi produce aflatoxins within crops. Thus, climate change has the potential to alter both the incidence and severity of aflatoxin contamination events.[91,95] Contamination is favored by hot and dry climates.[40,96,97] Hot climates also favor higher densities of *A. flavus* and higher incidences of the S strain[22,50] indicating periods of increased soil temperature drive selection of the highly toxigenic S strain.[91] Although certain crops apparently favor the S strain (Fig. 3), it is difficult to separate crop rotation influences from environmental influences. The S and L strains have different adaptations,[22,24,95]

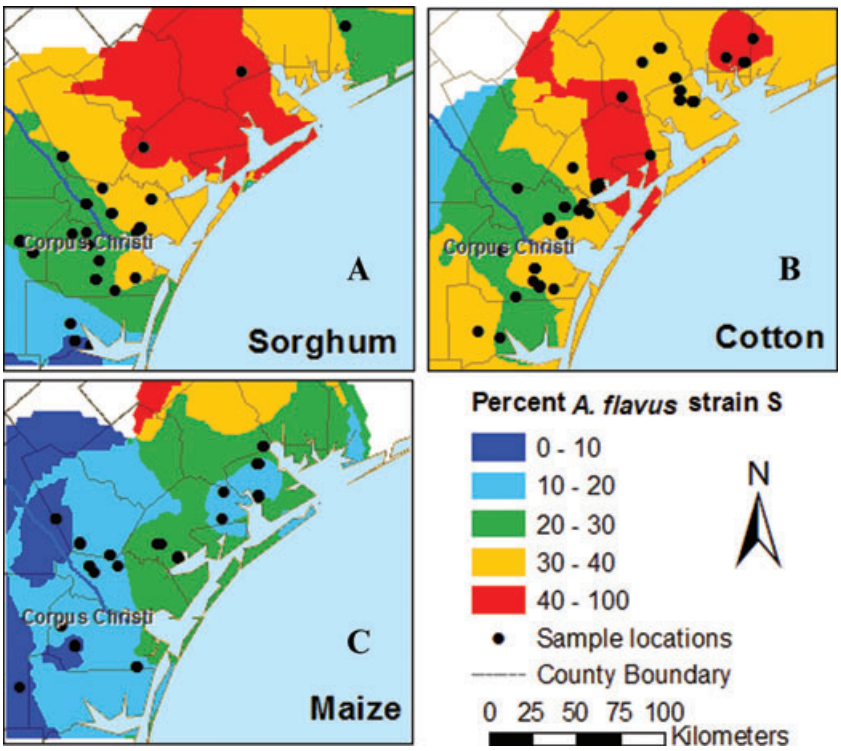

Figure 3. Percentage of the population of *A. flavus* composed of the S strain in soils of southern Texas previously cropped to sorghum (A), cotton (B), and maize (C). Rotations with sorghum and cotton favored increased incidences of the high aflatoxin-producing S strain morphotype. Rotation to corn favored reduced S strain incidence and increased frequences of the L strain morphotype that has lower average aflatoxin-producing potential. Maps of data previously reported in Ref. 74.

with the S strain better adapted to crops grown in warm environments. S strain isolates are most prevalent in warm regions of western and central Arizona and southern Texas where cotton and sorghum are major crops.[15,50,74,75] However, aflatoxin producers with S strain morphology also dominate in portions of East Africa[14] and in northern Texas where maize is an important crop. Repeated and severe contamination in eastern Kenya is due in part to high incidences of isolates with S strain morphology.[3,14] Thus, temperature influences on prevalence of these fungi may be a mechanism through which climate change will threaten food safety and human health worldwide.[91,95]

Conclusions: Future directions for biological control of aflatoxin-producing fungi

There are many genetically diverse atoxigenic VCGs of *A. flavus*, and atoxigenic isolates have been found in every target region examined to date.[4,15,44,49] In many regions, there are sufficient endemic, well-adapted atoxigenic strains to permit treatment with complex strain mixtures and to rotate mixtures between seasons and crops. This strategy has the potential to allow for additive and long-term reductions in the aflatoxin-producing potential of *A. flavus* communities associated with crops and throughout the environment and, in so doing, elimination of frequent human exposure to unsafe aflatoxin concentrations. As described in this review, phenotypic and genotypic variation among *A. flavus* individuals confers differential adaptation to hosts, soils, and climate. These divergences influence abilities of individual atoxigenics to compete across landscapes and through crop rotations. Currently, changes to fungal community structures caused by atoxigenic strain application gradually decline over three years.[17,98] Improving persistence of applied atoxigenic biocontrol strains may be possible through the selection of agroecosystem-adapted strains, use of strain mixtures that include isolates adapted to different niches/hosts or environmental fluctuations within the agroecosystem, and the implementation of agronomic practices that favor atoxigenics and suppress highly toxigenic aspergilli (i.e., the S strain). The future of atoxigenic strain technology should include assessment of phenotypic and genotypic diversity within *A. flavus* populations in order to develop formulations containing mixtures of atoxigenics with adaptive traits that will allow for long-term residence in target regions, thus increasing protection from aflatoxins at reduced cost.

Conflicts of interest

The authors declare no conflicts of interest.

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