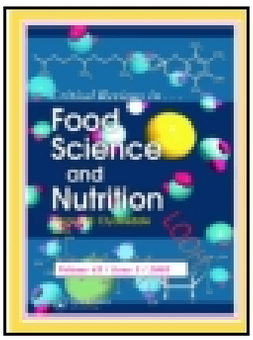

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REVIEW

Practical considerations will ensure the continued success of pre-harvest biocontrol using non-aflatoxigenic *Aspergillus flavus* strains

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ABSTRACT

There is an important reason for the accelerated use of non-aflatoxigenic *Aspergillus flavus* to mitigate pre-harvest aflatoxin contamination … it effectively addresses the imperative need for safer food and feed. Now that we have decades of proof of the effectiveness of *A. flavus* as biocontrol, it is time to improve several aspects of this strategy. If we are to continue relying heavily on this form of aflatoxin mitigation, there are considerations we must acknowledge, and actions we must take, to ensure that we are best wielding this strategy to our advantage. These include its: (1) potential to produce other mycotoxins, (2) persistence in the field in light of several ecological factors, (3) its reproductive and genetic stability, (4) the mechanism(s) employed that allow it to elicit control over aflatoxigenic strains and species of agricultural importance and (5) supplemental alternatives that increase its effectiveness. There is a need to be consistent, practical and thoughtful when it comes to implementing this method of mycotoxin mitigation since these fungi are living organisms that have been adapting, evolving and surviving on this planet for tens-of-millions of years. This document will serve as a critical review of the literature regarding pre-harvest *A. flavus* biocontrol and will discuss opportunities for improvements.

KEYWORDS

A. flavus; fungal sex; meiotic recombination; biocontrol stability; mycotoxins; mechanisms of control

Introduction

Even though aflatoxins have been an agricultural problem for many of the decades preceding their discovery and identification, they were not part of global public consciousness until the outbreak of Turkey X disease in the early 1960s (Blount 1961; Stevens et al. 1960; Van Der Zijden et al. 1962). Over the following 60 years, research has explored various aspects of aflatoxins, identified and described the fungi that produce them, and worked to develop strategies that mitigate their negative impacts on food and feed safety from both economic, and human and animal health perspectives. This document will provide an overview of the literature encompassing aflatoxins, aflatoxin-producing fungi and various aflatoxin mitigation strategies. Primarily, it will serve as a critical review of the literature surrounding the increasing use of non-aflatoxigenic *A. flavus* strains as biological control (biocontrol) and highlight the need for practical considerations for its improvement.

An overview of 60 years of Aflatoxin-Related research

Aflatoxins are toxic secondary metabolites that may exist in as many as 18 forms and derivatives, including B-aflatoxins (B_1, B_2, B_{2a}), G-aflatoxins (G_1, G_2, G_{2a}) and M-aflatoxins (M_1, M_2) (Benkerroum 2020). B-aflatoxins are so-named because they fluoresce blue when exposed to long-wave (365 nm) ultraviolet (UV) light, and B_1 is considered the most potent mycotoxin ever discovered, while G-aflatoxins fluoresce green and G_1 is secondary in its potency (Ambrecht et al. 1963; Klich 2007). B-aflatoxins have several hydroxylation products that include M-aflatoxins (Yabe et al. 2012). M-aflatoxins were originally reported to derive during production of milk from cows that ingested aflatoxin contaminated feed (De Iongh et al. 1964), and have since been detected in other mammalian biological fluids (Benkerroum 2020; Yabe et al. 2012), but their recent detection in *A. flavus* cultures means they are inherent in some fungal strains (Uka et al. 2019). Although no characterization yet exists of the conversion pathway from B- to M-aflatoxin production, both B- and G-aflatoxins are known to be synthesized by enzymes from a pathway of 25 clustered genes, most of which have been functionally characterized (Yu, Bhatnagar, and Cleveland 2004; Yu et al. 2004). There are several methods to detect the presence of aflatoxins, and each with its own set of potential limitations for accuracy (Gell and Carbone, 2019). Part of the problem with detecting aflatoxin production is the inconsistency of production on different substrates. A strain that produces low concentrations of aflatoxin on one substrate may produce high concentrations on a different substrate.

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We still do not know why *Aspergillus* microfungi (i.e. microscopic fungi) produce aflatoxins, nor any of the myriad of other toxic secondary metabolites they produce in conjunction with aflatoxin. Microfungi are nothing if not efficient biofactories (Hyde et al. 2019), so these metabolites likely serve multiple purposes. One suggested purpose for aflatoxins is as attractants to facilitate insect herbivory of plant tissues, thereby allowing these weakly pathogenic fungi to more easily invade the host and facilitate dispersal of conidiospores, especially in soil environments (Ehrlich 2006). Other suggestions include repellents that deter competing microbes from infecting host tissues as well as deterring insect fungivory (Drott et al. 2017), or protectants from UV damage (Ehrlich 2006) or oxidative stress (Reverberi et al. 2012). These compounds might also be involved in quorum sensing, including as stress response mechanisms during infection or drought stress in plants (Affeldt, Brodhagen, and Keller 2012; Sweany and Damann 2020). Whatever their purpose, aflatoxins pose a serious threat to our economy and health.

From an economic perspective, aflatoxins are a global concern. The preferred hosts for aflatoxigenic Aspergilli include some of the more important agricultural commodities, such as corn, cotton, peanuts, rice and tree nuts (Rushing and Selim 2019). Most of these crops produce seeds that are rich in oil (lipid) content, and lipids are what is consumed (Gao and Kolomiets 2009). Therefore, the higher the lipid content, the higher the amount of aflatoxin produced (Fountain et al. 2014). Yield loss not only means less consumable food or feed, it also means economic loss for growers. Each year, the aforementioned crops offer billions of dollars' worth of economic incentive to global markets to protect them from aflatoxin contamination (Pitt et al. 2012; Schmale and Munkvold 2009).

The health perspective in relation to aflatoxins is more of a concern in low to middle income countries (LMICs) who have not yet set, or do not adhere to, strict guidelines for acceptable levels of aflatoxins in foods and feeds, while several high income countries have strict guidelines in place (Pitt et al. 2012; Rushing and Selim 2019). China and some African countries have recently set stricter limits to lessen the effects of aflatoxin exposure (Chinese Pharmacopeia Commission 2015; Okoth 2016). The negative impacts of aflatoxins are mostly the result of ingestion, termed aflatoxicosis or aflatoxin poisoning (Dhanasekaran et al. 2011), although reports exist of aflatoxin B_1 being absorbed through the skin (Boonen et al. 2012; Rastogi et al. 2006; Riley, Kemppainen, and Norred 1985). Aflatoxicoses result when cytochrome P450s in the body convert aflatoxins into reactive forms that bind to DNA in liver tissue, as well as albumin in blood that gets diverted to the liver, and are mostly chronic (long-term exposure), but sometimes acute (short-term exposure) (Bbosa et al. 2013). Africa has experienced nearly 20 aflatoxicosis outbreaks between 1960 and 2016, with most of the outbreaks occurring in Kenya (Okoth 2016). In the United States, two reported episodes of lethal animal aflatoxicoses occurred when dogs had consumed aflatoxin-contaminated food (Bingham et al. 2004; Lightfoot and Yeager 2008). As recently as September of 2020, a dog food recall occurred in Louisiana due to potential contamination with aflatoxin. Other potential health risks from aflatoxin exposure include reduced growth, immune suppression and nutritional deprivation (Okoth 2016; Rushing and Selim 2019). The locations most at risk of aflatoxicosis usually have human and animal populations with barely enough food or feed to sustain them, therefore consuming contaminated food is preferred to starvation (Azziz-Baumgartner et al. 2005). Similarly, post-harvest storage of food or feed in conditions favorable to growth of aflatoxigenic fungi perpetuates exposure risk to aflatoxin poisoning once the food or feed is consumed (Alsuhaibani 2018; Benkerroum 2020). A compounding problem involves compromised immune systems. Healthy individuals are less prone to aflatoxicosis issues, but many people in LMICs (e.g. Southeast Asia and Africa) are malnourished and may have other underlying health problems such as hepatitis (a liver-taxing condition) that render them incapable of tolerating aflatoxin exposure (Benkerroum 2020; Magnussen and Parsi 2013). With the added potential for *A. flavus* to be pathogenic of immune-compromised humans and animals (e.g. aspergillosis) (Pasqualotto and Denning 2008), the risks from exposure to *A. flavus* fungi increase.

Pietro Antonio Micheli first described the *Aspergillus* genus in 1729 (Schaechter 1999). *Aspergillus flavus* is the more infamous producer of aflatoxins, and the species from which the term "aflatoxin" (*A. flavus* toxin) is derived (Austwick and Ayerst 1963). It was first described by J.H.F. Link in 1809 (Link 1809). Other *Aspergillus* species may produce one or more forms of aflatoxins, and most of these aflatoxigenic species are in the genus' Section *Flavi* (Frisvad et al. 2019). Additionally, many are of agricultural importance due to their association with food and feed commodities. Not all species within Section *Flavi* produce aflatoxins, such as *A. caelatus*, *A. oryzae, A. sojae* and *A. tamarii* (Frisvad et al. 2019). Similarly, not all *A. flavus* strains produce aflatoxins (i.e. non-aflatoxigenic), being genetically inhibited through naturally occurring mutations in, or loss of, genes from the aflatoxin pathway (Chang, Horn, and Dorner 2005; Chang et al. 2012; Ehrlich and Cotty 2004; Moore et al. 2009). Mycotoxins produced in conjunction with, or in lieu of, aflatoxins may include aflatrem, aspergillic acid, aspirochlorine, cyclopiazonic acid (CPA), kojic acid, sterigmatocystin and *O*-methylsterigmatocystin (Chankhamjon et al. 2014; Frisvad et al. 2019; Uka et al. 2019; Valdes, Cameron and Cole 1985). The latter two mycotoxins happen to be the respective penultimate and ultimate precursors to aflatoxin in the biosynthesis pathway (Carbone et al. 2007b). For example, mycotoxins have been confirmed in non-aflatoxigenic *A. caelatus* (e.g. aspirochlorine and tenuazonic acid), *A. oryzae* (e.g. aspirochlorine, CPA and penicillin), *A. sojae* (e.g. aspirochlorine and chrysogine), and *A. tamarii* (e.g. aspirochlorine, CPA and tenuazonic acid) (Frisvad et al. 2019). Goto and coworkers (1996) found *A. tamarii* strains that produced aflatoxin and CPA, but later re-classified them as *A. pseudotamarii* (Ito et al. 2001). Despite their potential to produce mycotoxins other

than aflatoxins, many consider *A. oryzae* and *A. sojae* as safe, used for hundreds of years in food and drink fermentation in East Asian countries (Jørgensen 2007; Machida 2002). These two species are respective domesticated ecotypes of *A. flavus* and *A. parasiticus* (Frisvad et al. 2019; Rokas et al. 2007), the result of serial culturing for many hundreds of years. Serial culture of a mycotoxigenic *Aspergillus* strain can cause it to lose the ability to produce one or more toxic secondary metabolites (Horn and Dorner 2002). *Aspergillus flavus* is capable of producing many different mycotoxins besides aflatoxin (Frisvad et al. 2019). Recently, Uka et al. (2017) reported several types of CPA possible in *A. flavus*, with the most serious of them being α-CPA. Another report suggested that a decrease in aflatoxin production results in an increase in CPA production (Vaamonde, Patriarca and Pinto 2006). A study by Moore et al. (unpublished data) showed substantial increases in CPA production in *A. flavus* strains, coinciding with significant reduction of their aflatoxin levels. In addition to being a mycotoxin, one report suggested that CPA is a potent pathogenicity factor for *A. flavus* (Chalivendra et al. 2017). Lack of regulation of the other *Aspergillus* mycotoxins suggests that any economic and health burdens they may impose are negligible, although CPA may have contributed as much as aflatoxin to poult deaths during the Turkey X disease outbreak (Bradburn, Coker, and Blunden 1994; Cole 1986). Based on previous toxicology tests, one study indicated that human intake of CPA should not exceed 10 ppb per day (Burdock and Flamm 2000).

The early stages of aflatoxin control involved cultural practices (e.g. crop rotations, quality seed selection, or altered planting schedules) and/or chemical fungicides to prevent growth and colonization by aflatoxigenic fungi (Rajasekaran, Cary, and Cleveland 2006). Some of these are still important practices that come with their own sets of drawbacks, especially the use of chemical fungicides for food protection since they are not environmentally sound, potentially harmful to consumers of treated foods and feeds, and the target fungi can become resistant (Nicolopoulou-Stamati et al. 2016; Reis et al. 2020). In the 1970s, post-harvest commodity treatments with ammoniation proved successful at reducing aflatoxin levels (Brekke, Peplinski, and Lancaster 1977; Gardner et al. 1971). Even post-consumption treatments involving materials that bind aflatoxins in the body have shown promise (Afriyie-Gyawu et al. 2007; Phillips et al. 2008). Over time, exploration and implementation of other strategies such as conventional breeding for inheritance using naturally resistant crop varieties, and genetic engineering of plants to increase their resistance, have been undertaken (Rajasekaran, Cary, and Cleveland 2006). The former is very time consuming, and the latter is not so easily embraced by a human population whose majority fears genetically modified foods. Research into biological control of plant pathogens using fungi such as *Trichoderma* began as early as the 1930s (Weindling 1932). Since then, biocontrol strategies to inhibit *aflatoxin* producing fungi and/or degrade aflatoxins have included exposure to plant-derived antifungal compounds (Loi et al. 2020), competing microbes such as the bacteria *Bacillus* and *Streptomyces* (Nesci, Bluma, and Etcheverry 2005; Palumbo, Baker, and Mahoney 2006; Shakeel et al. 2018), or fungi such as *Candida* and *Pichia* yeasts (Hua, Baker, and Flores-Espiritu 1999; Jaibangyang, Nasanit, and Limtong 2020; Moradi et al. 2020) and *Trichoderma* (Calistru, McLean, and Berjak 1997; Kifle, Yobo, and Laing 2016). These types of studies continue and have been the subject of previous reviews (Mannaa and Kim 2016, 2017; Ren et al. 2020), but many of these microbes have only exhibited success in the laboratory. The most promising strategy involves the use of naturally non-aflatoxigenic *A. flavus* strains as biopesticides (Abbas et al. 2011; Bandyopadhyay et al. 2016; Mauro et al. 2018). It should be noted that a holistic approach to aflatoxin control, involving multiple strategies, is likely the best long-term means of addressing the aflatoxin problem since there is no "silver bullet" (Cary et al. 2011; Ojiambo et al. 2018; Soni et al. 2020; Udomkun et al. 2017; Unnevehr and Grace 2013).

The history and evolution of non-aflatoxigenic A. flavus *as biocontrol*

In the late 1980s, the story of the most effective form of aflatoxin mitigation began when an *A. flavus* strain, sampled in an upland cotton field in Yuma, Arizona, showed itself to be naturally incapable of producing aflatoxin. Named AF36, lab tests showed it to be an aggressive colonizer that was able to out-compete and greatly mitigate aflatoxin production (Cotty 1989, 1994). The potential was then uncovered for this strain to be a safer alternative to chemical fungicides as an agent of biocontrol. A later investigation into the cause of its non-aflatoxigenic phenotype showed a single mutation in the *pksA* (*aflC*) gene of its aflatoxin pathway, which introduces a premature stop codon and renders it defective. Thus, it prevents downstream synthesis of any intermediate compounds that contribute to aflatoxin production (Ehrlich and Cotty 2004). At a similar time point as the discovery of AF36, another non-aflatoxigenic *A. flavus* strain (NRRL 21882), sampled in a Georgia peanut field, also exhibited an aggressive nature against native aflatoxigenic strains in laboratory tests, and showed promise as a potential biocontrol agent (Dorner, Cole, and Blankenship 1992). A formulation of NRRL 21882 was developed and named Afla-Guard® for use in peanut fields in and around Georgia (Dorner and Lamb 2006). The cause of its non-aflatoxigenic phenotype is the complete absence of its aflatoxin gene cluster (Chang, Horn, and Dorner 2005). Other non-aflatoxigenic strains that lack some or all of the aflatoxin cluster genes have been sampled and characterized (Chang, Horn, and Dorner 2005; Savić et al. 2020), and have been suggested to form a distinct, non-aflatoxigenic lineage for *A. flavus* that has experienced gene loss (Moore et al., 2009). Field trials for both AF36 and Afla-Guard® were conducted throughout the 1990s, and by the early 2000s both were EPA-approved for conditional use as pre-harvest biopesticides (USEPA 2003, 2004a). It is not uncommon to observe greater than 50% reduction in field levels of aflatoxins from

pre-harvest application of these biocontrol strains, some of which may continue into post-harvest (i.e. storage) (Abbas et al. 2006; Atehnkeng et al. 2014; Dorner and Cole 2002; Dorner, Cole and Wicklow 1999; Savić et al. 2020); however, the consistency of suppression is not guaranteed for every crop, or from use of every non-aflatoxigenic strain, and it is highly dependent on post-harvest conditions (Kingyungu et al. 2019; Seetha et al. 2017). Based on their successes, AF36 Prevail® is now widely used in the western part of the USA on aflatoxin-susceptible crops such as corn, cotton, almonds, figs and pistachios (Ortega-Beltran et al. 2019; USEPA 2017). Syngenta Corporation adopted the Afla-Guard® license in 2009 for use on corn and peanuts grown in the eastern part of the USA (Regnault-Roger 2012), and has since tested it on crops in other countries. For example, in Turkey this biopesticide was tested for prevention of aflatoxin contamination in peanut (Lavkor et al. 2019), and a study in Brazil reported concomitant reduction in *Fusarium verticillioides* and fumonisin contamination in maize from its use (Reis et al. 2020). Another candidate biocontrol strain in the United States, known as K49, was isolated from Mississippi corn (Abbas et al. 2006). It has the same aflatoxin inhibiting mutation in its *aflC* gene as AF36, and both strains share a vegetative compatibility group (VCG), but they are not the same strain (Chang et al. 2012). It has proven effective at reducing aflatoxin levels in field trials (Abbas et al. 2011), but at this time it is not a commercial biopesticide.

The efficacy of these non-aflatoxigenic strains has motivated more research and development of other candidate biocontrol strains around the world (Camiletti et al. 2018; Mallikarjunaiah et al. 2017; Mamo et al. 2018; Pitt and Hocking 2006; Tran-Dinh, Pitt, and Markwell 2014). A commercially available biopesticide developed in Italy (AF-X1™) is helping to save maize crops used for animal feed (Mauro et al. 2018). The strain used for the Italian biopesticide formulation (A2085) is similar to NRRL 21882 with regard to lacking the aflatoxin gene cluster (Mauro, Battilani, and Cotty 2015). Another biocontrol strain reportedly successful at reducing aflatoxin in maize for use in Serbia is MyToolBox Af01. A partial-cluster strain, it lacks aflatoxin cluster genes from *aflT* to *aflN* (Savić et al. 2020).

All of the biopesticide formulations mentioned above are comprised of a single non-aflatoxigenic strain used to inundate a field, yet the technology is evolving. Composite biocontrol formulations now exist that include more than one non-aflatoxigenic strain. The first composite biopesticide was created for use in Africa, where allowable aflatoxin levels are not well regulated and aflatoxin continues to be a source of major economic and health issues for growers and consumers (Atehnkeng et al. 2014). Initially developed for use in Nigeria, this formulation (called AflaSafe®) contains four different non-aflatoxigenic strains that derive from different VCGs, and the genetic bases for their non-aflatoxigenic phenotypes include point mutations in, or lack of, aflatoxin cluster genes (Atehnkeng et al. 2014). AflaSafe® is now a trade name under which many composite formulations exist for use throughout Africa (Bandyopadhyay et al. 2016). In Texas, a composite of four non-aflatoxigenic strains has been promoted as an effective biocontrol formulation for corn called FourSure™ (Moral et al. 2020; Shenge, Mehl, and Cotty 2017). A very recent study from North Carolina State University has also suggested use of a composite biocontrol formulation including at least two non-aflatoxigenic *A. flavus* strains (Molo et al. 2019). The use of non-aflatoxigenic *A. flavus* as biocontrol is evolving in its application, but in the course of its utilization, there has been little interest in exploring how it could fail us and how we can preemptively minimize any risks.

Practical considerations necessary for the future of *A. flavus* biocontrol

Fungi are highly adaptive organisms, especially under constant selective and environmental pressures (Naranjo-ortiz and Gabaldón 2019; Read 2007; Rédou et al. 2016). We are in a constant arms race with pathogenic microbes, including aflatoxin producing *Aspergillus* species such as *A. flavus* (Grandaubert, Dutheil and Stukenbrock 2019; Maor and Shirasu 2005). Therefore, we must work to understand more completely these fungal pathogens, and to improve biocontrol strain selection and use, through practical considerations. Current biocontrol formulations have proven to be highly effective at reducing aflatoxin levels, which is a boon to the economy and to consumer health. However, because this strategy is not a silver bullet we must remain cognizant of its caveats and anticipate *possible* issues that we can proactively address so they never become *real* issues.

How "atoxigenic" is the biocontrol strain?

Biocontrol strains are often referred to as "atoxigenic" (Bandyopadhyay et al. 2016, 771; Grubisha and Cotty 2015, 5889; Mauro et al. 2018, 1; Moore et al. 2015, 301; Ortega-Beltran et al. 2019, 905; Weaver and Abbas 2019, 1), "non-toxic" (Cole and Cotty 1990, 63), or "non-toxigenic" (Cole and Cotty 1990, 64; Dorner 2009; Gasperini et al. 2019) when they are *non-aflatoxigenic* at best (King et al. 2011). Their inability to produce aflatoxins does not mean non-aflatoxigenic fungi are incapable of producing other mycotoxins (Kagot et al. 2019). Earlier it was mentioned that *A. flavus* has potential to produce other mycotoxins such as CPA. The AF36 biocontrol strain has a functional CPA gene cluster, and readily produces α-CPA, as well as other CPA-like compounds, precursors and derivatives (Uka et al., 2017). Gene expression studies indicate it also has potential to produce kojic acid and aflatrem (Fountain et al. 2016). Even NRRL 21882, which lacks the CPA gene cluster, produces several CPA-related compounds, suggesting the potential for heterokaryosis in this strain (Uka et al., 2017). This strain also expressed the genes attributed to kojic acid and aflatrem production (Fountain et al. 2016), and it does produce low levels of kojic acid, which is not a regulated mycotoxin at this time (USEPA 2004b). K49 has a point mutation in the *pks-nrps* gene of its CPA cluster that renders it incapable of producing this mycotoxin (Chang et al. 2012). This

is one difference distinguishing it from AF36 although they share a VCG. However, its potential to produce the other CPA derivatives, or any other mycotoxins, has yet to be determined. Whether or not the other strains tested or used for biocontrol throughout the world have been assayed for their potential to produce mycotoxins beyond aflatoxins and CPA is unknown.

In other words, there is likely never a case where an *A. flavus* strain is completely atoxigenic. Many reports appear to use the two terms interchangeably, as if they mean the same thing. Perhaps it is because "atoxigenic" is a more concise word than "non-aflatoxigenic"… fewer characters to repeatedly type. No matter the reason, this inaccurate designation, especially when marketing a strain for biocontrol use, may inadvertently mislead growers and consumers into thinking there are no mycotoxins present. Some might argue that any other mycotoxins produced by a non-aflatoxigenic strain are not worth considering, given the overwhelming severity of aflatoxins. They may not be considering aggregate or synergistic effects of seemingly less severe *Aspergillus* mycotoxins. For example, an additive effect could result from inundating a field with copious quantities of a strain that produces even low-levels of CPA (King et al. 2011). Alternatively, there may be a synergistic effect of CPA (or any other mycotoxin produced by a non-aflatoxigenic strain) with mycotoxins produced by one or more other species (i.e. *Fusarium* or *Penicillium*) occupying the same niche (Bernhoft et al. 2004; Klarić, Rašić, and Peraica 2013; Sobral et al. 2018; Widiastuti et al. 1988).

Uka et al. (2019) examined over 50 *A. flavus* strains for the production of 50 toxic secondary metabolites, and none of the strains were atoxigenic. In fact, very few strains lacked production of all but one or two toxic metabolites. Neither of the commercially available biocontrol strains (AF36 or NRRL 21882) produced less than 11 toxic secondary metabolites. The growth substrate for their strains was Wickerham medium, and since metabolite production can be substrate dependent (Greeff-Laubscher et al. 2020), there is no guarantee it would produce the same metabolites on host tissue, or another synthetic medium used for aflatoxin assays such as Glucose-Mineral Salts (GMS) or Yeast Extract Sucrose (YES).

Therefore, we should cease use of "atoxigenic" without proof, and consider and assess all potential mycotoxins produced by current biocontrol strains, as well as their possible synergistic or additive effects. This way, growers make an informed decision before selecting, purchasing and repeatedly utilizing a biocontrol formulation that they assume produces no mycotoxins. At the very least, researchers should screen for all potential mycotoxins in candidate biocontrol strains and then select those who lack production of as many toxic secondary metabolites as possible. This protects consumers from unknowingly ingesting any mycotoxins that remain on the food or feed crop and possibly pose a compounded risk to their health.

How ecologically stable is the biocontrol strain?

There is a recommendation to apply biocontrol strains every year, even if environmental conditions are not conducive to an aflatoxin outbreak (Abbas, Accinelli, and Shier 2017; Ehrlich 2014), which seems an unnecessary burden on growers if biocontrol strains are stable beyond one growing season (Bandyopadhyay et al. 2016). One reason for this recommendation is to account for decrease in biocontrol numbers into subsequent growing seasons (Grubisha and Cotty 2015), which can occur if weather conditions after field release of biocontrol are not conducive to its survival (Pitt 2019). This recommendation is also to create an additive effect over multiple growing seasons (Cardwell et al. 2015). An important reason annual application may be necessary is the potential for aflatoxin levels to return in treated fields, even if it may take more than a year (Weaver and Abbas 2019). Studies have reported an increase of post-harvest aflatoxin levels during storage and processing (Atehnkeng et al. 2014; Kinyungu et al. 2019). This suggests a maintained presence of toxigenic strains that develop or somehow regain production of aflatoxin as soon as conditions are favorable, so perhaps *A. flavus* biocontrol temporarily turns off aflatoxin production instead of outright displacing toxigenic strains. It also requires that candidate biocontrol strains be tested for their ability to maintain control over aflatoxigenic strains under varied storage conditions involving fluctuations in temperature and available water (Gasperini et al. 2019).

Studies indicate that biocontrol strains do persist beyond the year they are applied (Abbas, Accinelli, and Shier 2017; Bandyopadhyay et al. 2019; Doster, Cotty, and Michailides 2014; Weaver and Abbas 2019). In fact, Weaver and Abbas (2019) found no evidence to support the annual reapplication recommendation. Therefore, it appears growers may be able to wait at least one year between biocontrol applications, which would save them time and money. In their 4-year field tests, Weaver and Abbas used annual and biannual biocontrol treatments and observed persistence of applied strains as well as movement of biocontrol into neighboring untreated plots. However, they did not treat a field plot in year one and forego reapplication for the remaining three years, which would have been a worthy investigation to assess longer ecological stability.

Often, persistence of biocontrol strains is assessed through molecular analysis, but this is only an effective means of detecting the persistence of a biocontrol strain when it involves multiple loci. For example, if one applied AF36 to a field and then assessed persistence based only on the *aflC* gene's mutation as found in AF36, one might overlook a strain like K49, which has the same *aflC* mutation but is not the same strain. Likewise, to look only for the absence of a single gene to confirm the presence of NRRL 21882, one would need to consider a late-pathway gene since they might actually recover partial cluster strains lacking only a few early pathway genes (Kinyungu et al. 2019). So at best, single locus assessments of a biocontrol strain may only recover another non-aflatoxigenic strain… not the exact biocontrol strain used. With regard to field-level recombination, this same argument applies. It could be suggested that recombination is not occurring because there are plenty of isolates sampled with the one *aflC* mutation or are

missing any gene from the aflatoxin cluster. However, there is an entire genome potentially shuffled, so one cannot definitively say that recombination rates are low unless a multi-gene examination is conducted that covers multiple chromosomes.

One possible time- and cost-saving method to circumvent any biocontrol persistence issues would be to predict when and where aflatoxin outbreaks will occur. An accurate modeling system could alert growers of eminent aflatoxin-favorable conditions. At this time, the United States does not yet have a modeling system in place to predict when an aflatoxin outbreak may occur, and so the recommendation of annual reapplication is a way to be proactive in the face of uncertainty. Particular weather conditions (e.g. high temperature and drought conditions) must exist for increased risk of an aflatoxin outbreak, which may not occur with consistency. Modeling systems for maize and pistachio in Europe have been developed and implemented (Battilani et al. 2013, 2016; Kaminiaris et al. 2020), and peanut and maize models have been developed for use in Australia and Kenya (Chauhan et al. 2010, 2015), so perhaps others can follow their lead to develop one for aflatoxin-susceptible regions in the United States. Weather systems across the globe may be too random for a modeling system to be wholly effective, but the potential benefit is worth the effort to explore. Another readily implemented tactic to promote persistence of a biocontrol strain is to use it in fields where it already exists naturally, because using a non-native strain, or one with limited distribution in the area, might hinder its ability to adapt and survive (Grubisha and Cotty 2015; Lewis et al. 2019).

Beyond the conditions that facilitate success of aflatoxigenic *Aspergillus* species to infect and contaminate crops, we must also consider unfavorable conditions, arising from changes in climate (i.e. environmental stressors), that may influence the behaviors of these fungi. Global temperatures are rising, and so areas that were once inhospitable to growth by plant pathogens like *A. flavus* may experience more infections. Conditions of severe drought are stochastic at present, but may become more commonplace with climate shifts. Drought stress makes plants more susceptible to contamination by aflatoxigenic fungi (Scheidegger and Payne 2003). Several studies have shown that increased CO_2 levels lead to increased aflatoxin production, and trigger differential expression of aflatoxin-related genes in *A. flavus* (Gilbert et al. 2016; Gilbert et al. 2017; Medina, Rodríguez and Magan 2014; Medina et al. 2015; Medina et al. 2017). However, assessment of the impacts of this apparently important environmental parameter on non-aflatoxigenic [biocontrol] *A. flavus* strains is just beginning. Gasperini and coworkers (2019) reported that non-aflatoxigenic strains do not share the same level of resilience to climate stressors, and need vetting for this ability in light of rise in global CO_2 levels and temperatures, as well as variable water availability within host tissue across developmental stages. Although it may be possible for non-aflatoxigenic strains to more easily adapt to climate stressors as they gradually manifest, we should test this sooner rather than later, especially since they may need to control aflatoxigenic strains that are producing much higher quantities of aflatoxins. As well, we should continue to anticipate and explore other possible hindrances to biocontrol efficacy such as their reproductive and genetic stability.

How reproductively stable is the biocontrol strain?

Historically, researchers considered *A. flavus* as asexual (anamorphic) because there had been no physical evidence of a sexual (teleomorphic) state (e.g. gametangia, fruiting bodies, ascospores) observed in the first 200 years since its characterization. It was in the late 1990s, with the advent of phylogenetics, that genomic evidence suggested a cryptic sexual state for *A. flavus* (Geiser et al. 1998). Geiser and coworkers reported a level of genetic discordance that was impossible to explain without meiotic recombination (i.e. sex) in nature. It prompted them to consider what impacts sex could have on burgeoning interest in using non-aflatoxigenic *A. flavus* strains as biocontrol. This study also sparked a renewed interest in exploring the potential for *A. flavus* (and other sclerotial fungi) to have teleomorphic states similar to *A. alliaceus* (Malloch and Cain 1972). An investigation by Ramirez-Prado et al. (2008) identified and characterized the mating-type (MAT) locus in species from section *Flavi*. They found two types of MAT genes present in an *A. flavus* population previously sampled from a field in Georgia (Horn and Greene 1995). Then, a study by Moore et al. (2009) found an extensive history of recombination in the aflatoxin gene clusters of the same Georgia *A. flavus* population (representing 44 VCGs and 27 multi-locus haplotypes) … far more than was found for an *A. parasiticus* population from the same field (Carbone et al. 2007a). These studies suggested some level of sexual recombination occurring within *A. flavus* populations, and mentioned the implications for biocontrol. However, without proof of a sexual state, there was no cause to question biocontrol stability.

After nearly 200 years as an asexual fungus, Horn and coworkers were the first group to prove that sex is possible for *A. flavus* through intraspecific laboratory mating experiments (Horn, Moore, and Carbone 2009). Using incubation parameters that were previously established for *Petromyces alliaceus* by McAlpin and Wicklow (2005), Horn et al. co-inoculated conidia of compatible strains on mixed cereal agar and incubated them in constant darkness for a long period of time (up to 6 months) at 30 °C. The parent strains used for the *A. flavus* pairings were from different VCGs, because those sharing a VCG were the same mating type (Horn, Moore, and Carbone 2009). All of these pairings resulted in variable production of cleistothecia, and nearly all of those cleistothecia bore ascospores. The sexual states of *A. flavus* and *A. parasiticus* (discovered around the same time) were given the genus name *Petromyces* (Horn, Moore, and Carbone 2009; Horn, Ramirez-Prado, and Carbone 2009). This was before the "One Fungus = One Name" movement pushed for the anamorph genus be used for most Aspergilli (Pitt and Taylor 2014; Taylor 2011; Taylor, Göker,

and Pitt 2016; Turland et al. 2018). Now both the sexual and asexual forms of all species in subgenus *Circumdati*, which includes section *Flavi*, are to use the asexual genus name of *Aspergillus*.

The finding that sex involving *A. parasiticus* and *A. flavus* strains occurs between individuals from different VCGs turned out to be an important finding, because under normal conditions anastomosis of strains from two different VCGs results in death of their fused cells. If the need to undergo sex relaxes the barriers of heterokaryon and vegetative incompatibilities, it must be important. What ecological or environmental parameter(s) might trigger the relaxation of these barriers and facilitate sex between strains of different VCGs? One suggestion relates to lack of fitness or the "abandon-ship" mechanism (Schoustra et al. 2010). For species like *A. flavus*, this could occur when wind or insects disperse spores well beyond their established habitats (Golan and Pringle 2017). These "foreign" strains may not be as well suited to the new environment and must attempt sexual recombination with local strains to improve their chances of survival. Another suggested trigger is environmental stress, which induces fungal sex in order to circumvent an unfavorable situation (i.e. adaptation) (Nieuwenhuis and James 2016).

So what could explain our inability to observe physical evidence of sex in the nearly 200 years we have known about *A. flavus*? Frequency could be a factor, given that we still do not know how often this phenomenon occurs in nature. Varying degrees of sexual recombination have been observed for global populations of *A. flavus* (Moore et al. 2009, 2013, 2017), but these likely related to another factor, which is mating type distribution in a field. Field populations that skew to one mating type over another or that are entirely composed of a single mating type experience very little to no sexual recombination (Moore et al. 2013; Ramirez-Prado et al. 2008). Timing could also be a factor. When the first laboratory mating experiments were conducted, the mating process was thought to be 6 months at least (Horn et al. 2009). Now we know that the entire process can occur in 5–8 weeks (Horn et al. 2014; Luis et al., 2020). This is a relatively short window to witness the sexual state in nature, especially when one does not know the exact moment the mating process begins, or what specific environmental conditions in the field trigger sex in *A. flavus*. Structural changes that occur when a sclerotium becomes a stroma could also be a factor (Luis et al., 2020). There is a channeling of the sclerotium's tightly packed mass of internal hyphae to make space for the developing cleistothecia (whose non-rigid walls easily break down), and the thickness of its exterior rind decreases as the cleistothecia enlarge, filling with asci and ascospores. These changes likely allow the stroma to break open more easily and release ascospores. If stromata form mostly on the soil surface, rapid breakdown from exposure to the elements or accidental crushing would render their persistence in the field short-lived. Additionally, since sexual recombination can occur in response to stress (Nieuwenhuis and James 2016; Usher 2019), perhaps the absence of fruiting bodies in sclerotia harvested from plant tissue relate to an abundance of nourishment provided by the host (e.g. cottonseed or corn kernels), thereby circumventing potential stressors for fungal strains. As well, if VCG and mating-type diversity are indeed lower in aerial host tissues compared to the soil environment (Sweany, Damann and Kaller 2011), the chances of finding a compatible mate in host tissues with whom to out-cross decreases.

Investigating sexual potential of A. flavus *biocontrol strains*

There have been several studies including AF36, NRRL 21882 and/or K49 as *MAT1*-2 parent strains to assess their fertility. Olarte et al. (2012) paired AF36 and NRRL 21882 with various *MAT1*-1 strains to test genotype and phenotype (e.g. aflatoxin and CPA) inheritance. There were four aflatoxin cluster regions examined including the *aflC* gene and three intergenic regions dispersed throughout the latter part of the aflatoxin pathway (from *aflG* to *aflX*). About 50% of the examined F1s from crosses involving AF36 were recombinants that inherited aflatoxin production, although only one of the examined offspring exhibited a mixture of cluster regions from both parents. The others inherited all four aflatoxin cluster regions from the toxigenic parent suggesting that most offspring inherit the entire aflatoxin gene cluster rather than an amalgamation of shuffled genes from both parents. However, without examining the entire pathway of genes, there can be no certainty that the offspring inherited the entire gene cluster. With regard to crosses involving NRRL 21882, 36% of the examined F1s inherited aflatoxin production, indicating they likely inherited the entire aflatoxin gene cluster from the toxigenic parent since it does not exist in NRRL 21882. An unexpected finding from this study was the amplification of cryptic alleles whereby five F1s amplified only one or two of the genomic regions from the aflatoxin gene cluster. In two of the five NRRL 21882 offspring, the defective *aflC* gene of AF36 amplified despite it not being one of the parents. In one of these two *aflC*-offspring, the *aflG/aflL* intergenic region was not from the toxigenic parent, nor was it the same as the AF36 parent. Olarte and coworkers stated the potential for cryptic heterokaryosis to exist in multi-nucleate *A. flavus*. Their findings support a previous study by Moore et al. (2009) whereby they unexpectedly amplified and sequenced the *aflW/aflX* intergenic region for NRRL 21882.

Horn and coworkers (2014) conducted a study whereby they mixed conidiospores of each biocontrol strain with a compatible (*MAT1*-1) toxigenic *A. flavus* strain and incubated them on mixed cereal agar until they formed sclerotia. In the lab, they then incubated some of the sclerotia on the surface of non-sterile soil and submerged some in non-sterile soil. Eight weeks of incubation were required to observe physical evidence of the sexual state, and sclerotia incubated on the soil surface contained greater numbers of fruiting bodies than submerged sclerotia. They determined that the soil surface is likely where out-crossing takes place, when sclerotia dislodge from aerial plant parts during harvest. Horn's group then conducted a study involving field incubation of sclerotia across three different locations in Georgia

(Horn et al. 2016). They placed lab-generated sclerotia on the soil surface in these fields, and left them for one year. This simulated what would occur in the field when sclerotia dislodge during corn harvesting and fall to the soil surface. Some of the sclerotia were from non-fertilized, individual strains (*MAT1*-1 or *MAT1*-2), and others were from fertilized co-cultures involving two sexually compatible strains (*MAT1*-1 + *MAT1*-2). They observed the *MAT1*-2 single-strain sclerotia (including the AF36 and NRRL 21882 biocontrol strains) yielding no cleistothecia or ascospores; however, both the non-fertilized *MAT1*-1 sclerotia and the fertilized *MAT1*-1 + *MAT1*-2 sclerotia had become stromata bearing fruiting bodies and ascospores. Examinations of allele inheritance revealed that progeny involving the non-fertilized *MAT1*-1 sclerotia inherited novel alleles from inherent soil fungi, and that progeny involving the fertilized sclerotia only inherited alleles from the original parent strains. Another finding from this study involved parental roles for conidia and sclerotia whereby they respectively act as male and female (i.e. hermaphroditic), so mitochondrial inheritance is only from the sclerotial parent. In all of these investigations into sex for *A. flavus*, the level of fertility was strain specific. We have learned that some *A. flavus* strains are incapable of undergoing sex (i.e. some are sterile). Horn et al. (2016) showed that in some strains only the conidia or the sclerotia exhibit fertility (i.e. not truly hermaphroditic), while others are more fecund because they out-cross through both their conidia and sclerotia. For example, both AF36 and NRRL 21882 were able to mate and contribute to production of ascospores; however, reciprocal crosses showed that only their conidia were fertile (their sclerotia appeared sterile). Therefore, field treatment with copious amounts of their conidia could fertilize inherent sclerotia from the native *MAT1*-1 population and potentially generate recombinant progenies. A laboratory study (Moore et al., unpublished data) paired the K49 biocontrol strain (*MAT1*-2; tagged with eGFP) with a wild-type *MAT1*-1 strain with proven fertility following the protocol of Horn, Moore and Carbone (2009). The goal was to look for evidence of recombination in F1 progenies by examining heritability of specific phenotypes (e.g. mycotoxins and fluorescence) and genotypes (e.g. MAT, aflatoxin gene cluster, and eGFP) based on sequencing the genomes of this familial population. The pairing was successful, and hundreds of ascospore progenies were harvested, even though only ten (five fluorescent and five non-fluorescent) were selected for further study. Their findings are forthcoming.

Because the process of mating for *A. flavus* and development of sexual structures occur inside the sclerotium, these phenomena could not be readily observed. As well, understanding the transition of a sclerotium to a stroma was lacking. A recent study by Luis et al. (2020) attempted to visualize the developmental process of sex in *A. flavus*. Using a mCherry-tagged *MAT1*-1 strain and a GFP-tagged *MAT1*-2 strain (NRRL 21882), they were able to distinguish hyphae from germinated conidia of one strain interlocking with hyphae from the other strains' sclerotia at their base as soon as 72 h of incubation. After five weeks of incubation, they observed green fluorescence and red fluorescence in fertilized sclerotia. By the eighth week, they observed differential development of Crozier's hooks, ascocarps, asci and ascospores, but no gametangia (ascogonia or antheridia) as previously observed for other Aspergilli (Benjamin 1955). Despite their success, some of the story surrounding sexual development in *A. flavus* remains untold.

There have also been interspecific mating experiments involving biocontrol strains. Olarte and coworkers (2015) successfully paired *A. flavus* and *A. parasiticus* strains, however none of their crosses involving a *MAT1*-1 *A. parasiticus* with *MAT1*-2 *A. flavus* (including AF36 and NRRL 21882) proved fertile. Three of the NRRL 21882 crosses yielded cleistothecia, but they were devoid of ascospores. One possible reason for the lack of hybrids from crosses involving biocontrol strains is unknown fertility. Of the seven *A. parasiticus* strains paired with either biocontrol strain, only three had been tested previously for their fertility and they were only moderately successful in producing intraspecific offspring (Horn, Ramirez-Prado and Carbone 2009). Another possible reason relates to the potential for infertility in conidia or sclerotia. It was not until after Olarte and coworkers completed their hybrid study that Horn and coworkers determined sclerotia of AF36 and NRRL 21882 to be sterile. This means that only their conidia were fertile and would require the sclerotia of the *MAT1*-1 *A. parasiticus* strains (with whom they were paired) to be fertile… if those *A. parasiticus* strains even produced sclerotia. A VCG study by Horn et al. (1996) explored sclerotium production by the same *A. parasiticus* strains used in the Olarte et al. hybrid study and found that most produced very few, if any, sclerotia. Production of sclerotia was tested by Horn et al. on Czapek's medium… not mixed cereal agar as was used for the mating studies. If Olarte and coworkers had conducted their study after Horn et al. reported their 2016 findings and consequently modified their strain selection, they could have observed inter-specific hybrid progenies resulting from out-crossing between each biocontrol strain and *A. parasiticus*. How many other aflatoxigenic species can potentially mate with biocontrol strains in the field? Only time, and more hybrid mating studies, will tell.

Implications of sexual recombination involving biocontrol strains

At the time this effective mitigation strategy was EPA-approved and commercialized, the assurance regarding an *A. flavus* biocontrol strain's ecological stability was its asexuality. That assurance is now an out-dated assumption, and ignoring it could affect biocontrol stability in the long-term. Despite the predominance of asexual propagation, positive selection maintaining the presence and functionality of MAT genes in fungi suggests that they continue to be necessary even if they are not frequently activated (Tsui et al. 2013; Wada et al. 2012; Wik, Karlsson, and Johannesson 2008). Even if the MAT locus is not itself undergoing positive selection but is linked to other loci that are, it is still functional in multiple aflatoxigenic fungi and benefits from the relaxation of such a strong barrier to genetic exchange

as vegetative incompatibility. Fungi that maintain both asexual and sexual states in their life cycles are less likely to experience an evolutionary dead-end like those who exhibit a solely homothallic (i.e. self-fertilizing) sexual state or who only reproduce asexually (Gioti et al. 2013; Taylor et al. 2015). *A. flavus* is a heterothallic fungus (Horn, Moore and Carbone 2009), which means it requires a genetically different partner to achieve maximum diversity during genetic exchange. There is no reason to discount that the predominant existence for *A. flavus* is asexual. It is far less taxing on fitness to propagate through production of copious amounts of conidiospores (Lee et al. 2010). Provided they are viable, even recombinant and hybrid strains will exhibit a predisposition for conidial propagation like their parents (Horn, Moore and Carbone 2009; Horn, Ramirez-Prado and Carbone 2009; Olarte et al. 2015). However, to state that sex and meiotic recombination are negligible in the field, or to disregard their potential impacts on field ecology, is to hinder the advancement or improvement of the biocontrol strategy. These phenomena do occur with much less frequency than asexual propagation, but they are far from negligible in terms of their influence on population diversity, secondary metabolite production and adaptation to environmental changes.

Ways to help ensure reproductive stability for biocontrol strains

There are reportedly seven criteria used to determine if a non-aflatoxigenic strain is worthy of inclusion as a biocontrol component (Moral et al. 2020), and currently none of those criteria assess fertility of the biocontrol strain. Several simple investigations could be undertaken relating to potential biocontrol strains that would confirm reproductive stability to lessen the chances of facilitating sex when inundating the field with biocontrol conidia and help ensure stability of the biocontrol strain in the field (Andrews et al. 2020). First, acknowledge that sexual recombination occurs in nature, no matter the frequency, and explore this potential with every candidate strain slated for biocontrol. Second, determine the presence and functionality of mating type genes in non-aflatoxigenic strains considered for biocontrol. Since the first characterization of MAT genes in *A. flavus* (Ramirez-Prado et al. 2008), the numbers of reports that openly disclose the mating type(s) of strains being considered for biocontrol formulations, or currently in use as biocontrol, are extremely limited. For example, reports involving the composite biocontrol formulations of AflaSafe and FourSure never disclose the mating types of the four non-aflatoxigenic strains that comprise each formulation (Agbetiameh et al. 2020; Atehnkeng et al. 2008, 2014; Bandyopadhyay et al. 2016; Senghor et al. 2020; Shenge, Mehl and Cotty 2017; Shenge et al. 2019; USEPA 2015). They only report the genetic conditions that predispose these strains to exhibit non-aflatoxigenic phenotypes. There are sequenced genomes for the FourSure strains one could search for MAT information (USEPA 2015), but they appear unavailable to the public via a repository like NCBI. Are they all of the same mating type? If so, this would ensure their inability to repair one another's non-aflatoxigenic genotype, but may not guarantee they would not recombine with field strains. If the MAT gene in one or more of these non-aflatoxigenic strains is missing or genetically broken, then there is even less chance for sexual recombination to occur among them or with the field population, which would enhance their reproductive stability. A concomitant investigation of MAT distribution should also occur for the field(s) where growers will use the biocontrol strains. In a field that is predominately of one mating type, simply ensure the biocontrol strain(s) is/are of the same (predominant) mating type (Moore et al. 2013; Ouko et al. 2018). In fields where the MAT distribution is equal, it would be beneficial to find and use a naturally infertile biocontrol strain (Moore et al. 2013; Ouko et al. 2018). Third, it is important to set up reciprocal mating experiments in the laboratory using the biocontrol strain's sclerotia or conidia to assess their fertility. Since mating experiments can complete in as little as 8 weeks, this could be undertaken while conducting an in vitro study of the strain's aflatoxin mitigation potential, or while undertaking time-consuming classical VCG testing. If biocontrol strains cannot produce offspring in the lab under controlled and specific environmental conditions, then it is even more likely they will not produce offspring in the field. Such simple acts as these could help ensure the reproductive stability of these important strains.

How genetically stable is the biocontrol strain?

An important way filamentous fungi maintain genetic stability is through a mechanism involving strain compatibility. *Aspergillus* strains form vast networks of haploid hyphae that frequently have contact with one another while sharing the same niche (Gould 2009). Their contact and fusion trigger a self-recognition system known as heterokaryon incompatibility, governed by a group of specific loci called *het* loci (Leslie 1993; Leslie and Zeller 1996; Papa 1986). Under normal conditions, the anastomosis of hyphae from two fungal strains offers two possible outcomes relating to heterokaryon incompatibility. When *het* loci are identical, the fused strains sense "self" and allow plasmogamy (i.e. the mixing of nuclei). Alternatively, when their *het* loci are not identical the strains enact a defense mechanism (cell death) to prevent the spread of potentially deleterious traits. Investigations into the identities and locations of *het* loci in *A. flavus* are relatively new research (Monacell et al. 2014; Mori et al. 2019). *A. flavus* reportedly contains 48 *het* loci (Mori et al. 2019); however, they are putative and still need confirmation.

VCGs are rooted in heterokaryon incompatibility (Leslie 1993; Leslie and Zeller 1996). For example, strains that share a VCG are identical at all of their *het* loci, although unique mutations in non-*het* loci may exist among them (Abbas et al. 2011; Chang et al. 2012). To maintain genetic stability, if two strains of the same species are not identical at all *het* loci, then they will not share a VCG and cannot exchange genetic material (Leslie 1993; Leslie and Zeller 1996).

Similarly, two strains from different fungal species will likely not share a VCG (Horn and Greene 1995). Therefore, under normal conditions the fused hyphal cell from strains of two different VCGs will die. The exact number of different *het* loci needed to segregate VCGs in *A. flavus* is unknown, but it may take the difference of only one *het* locus of the putative 48 to create a new VCG (Moore, unpublished data). *A. flavus* exhibits a high degree of diversity with regard to VCGs (Horn and Greene 1995; Islam et al. 2018; Ortega-Beltran and Cotty 2018). New VCGs within a field population can manifest at a slow rate through chance mutations that develop in *het* loci (Muirhead, Glass, and Slatkin 2002), but this alone would likely be reflected by low to moderate VCG diversity. Aside from the possible influx of foreign VCGs, diversity could arise at a faster rate through genetic recombination (Olarte et al. 2012).

The first description of genetic recombination possible for *A. flavus* occurred in the 1970s, referred to as parasexuality, which only occurs between essentially clonal strains from the same VCG (Leaich and Papa 1975; Papa 1973; Papa 1976). The frequency of parasexual recombination in nature is as unknown as that of sexual recombination, observed only in controlled environments, and yet some suggest parasexual recombination influences genetic diversity in *A. flavus* while meiotic recombination is negligible (Islam et al. 2018; Senghor et al. 2020). It may allow inherently identical fungi to adapt and evolve quicker than the manifestation of random mutations (Lee et al. 2010). However, even when random mutations arise in non-*het* loci, assuming a neutral mutation rate, the rate of evolution remains neutral as well.

Meiotic recombination shuffles the genomes of two different strains, from different VCGs, with one or more unique *het* loci. Sexual recombination is one way to create new VCGs, and *A. flavus* has historically exhibited high VCG diversity (Bayman and Cotty 1991; Horn 2003; Islam et al. 2018; Ortega-Beltran et al. 2019), and it seems the fastest way for a strain to adapt or evolve. Although sometimes detrimental for the recombinant offspring, there are certainly times when these genetic changes are advantageous and the recombinant individuals survive (Alves et al. 2017; Stapley et al. 2017). All of this considered, sex appears to remain an important life cycle component to maintain in these fungi.

Genetic stability is important in a biocontrol strain because it needs to maintain the non-aflatoxigenic phenotype (Ehrlich and Cotty 2004; Okoth et al. 2018). Since it is unlikely the genetic aberration causing a biocontrol strain's non-aflatoxigenic phenotype is repairable (in the biocontrol strain itself), the strain is genetically stable. This stability is more uncertain for offspring that could result from the biocontrol strain out-crossing with other individuals in a field population (Olarte et al. 2012). In other words, how heritable is the non-aflatoxigenic phenotype? Whether or not the genotype associated with non-aflatoxigenicity is heritable may depend on the type of genetic aberration and the likelihood of replacing aberrant or missing genes during meiotic recombination events. For example, a strain such as NRRL 21882 (lacking the aflatoxin and CPA gene clusters) is less likely to help produce mycotoxigenic offspring than a strain with full gene clusters, each containing one defective gene (e.g. AF36 or K49) that could be replaced with a functional gene during recombination (Olarte et al. 2012).

A very recent study (Gell, Horn and Carbone 2020) reported success at creating a genetic map for *A. flavus* that could help in identifying traits relating to sexual fertility and aflatoxin production, which could be used to optimize biocontrol strains and improve their sustainability. They also report that "many new *A. flavus* genotypes" are possible in a single generation resulting from sex between fertile strains, with up to 20 recombination events possible in a single progeny.

Considerations when sexual recombination is lacking in a population

When a study claims to find no evidence of recombination in its sample population, there may be several possible explanations. One relates to distribution of mating type genes in a population, making it possible to infer the presence or absence of recombination with a strong degree of confidence. Populations with disproportionate MAT distribution in favor of one mating type over the other will show little or no evidence of recombination (Moore et al. 2013). With a quick diagnostic PCR of pooled MAT primers, one can quickly assess this distribution and infer why recombination may or may not be detectable. Another explanation relates to VCG. If a study only involves strains that share a VCG, then the only type of recombination one could infer would be parasexual, considered rare in nature due to the near impossibility of its detection among inherently clonal strains (Clutterbuck 1996). Even if there are strains of each mating type sharing a VCG, sex and meiotic recombination will not be possible (Horn, Ramirez-Prado and Carbone 2009). If a study involves individual strains from different VCGs, the sample population size for those individuals must be large to capture evidence of meiotic recombination (Grünwald et al. 2017). Finally, simple sequence repeats (SSRs) on their own may not offer accurate inference of recombination among different VCGs since they can show a high degree of haplotype diversity for inherently clonal strains that share a VCG, and whose genes are shuffled parasexually at best. Microsatellite loci mutate rapidly, which can complicate inferences of population structure (Tsykun et al. 2017). They can also experience slippage (loss or gain of repeats) at replication with each generation (Heissl et al. 2019). Many generations are possible when *A. flavus* asexually propagates. If these repeats can inflate diversity for clonal strains that are identical at all 48 *het* loci, then on their own they cannot accurately assess the frequency of meiotic recombination among individual strains that are not identical at all *het* loci.

How does the biocontrol work?

We still do not fully understand the mechanism(s) by which biocontrol strains elicit control over native aflatoxigenic strains, but it works and that seems good enough for most

people. Competitive displacement or exclusion has been suggested as a possible mechanism (Atehnkeng et al. 2008; King et al. 2011; Pitt 2019). This insinuates that the indigenous toxigenic strains are no longer present in the population. Yes, it is expected that post-inoculation one finds much higher quantities of the biocontrol strain on the host, but when toxigenic strains can bring back aflatoxin levels in storage from a process as simple as rewetting (Kinyungu et al. 2019) the field load of toxigenic strains has not been displaced. In this case, the toxigenic strains are more likely outnumbered and their aflatoxin production has been quelled or turned off… something temporary. Another proposed mechanism of biocontrol is inhibition of aflatoxin production through physical contact or thigmoregulation (Huang et al. 2011). This seems a logical mechanism, however there were caveats associated with thigmoregulation success. For example, contact with the biocontrol strain had to occur within 24 h of a toxigenic spore's germination, and aflatoxin mitigation was not consistently observed for all toxigenic strains tested. Most recently, there has been study of chemical control as a mechanism involved in biocontrol success. Moore and coworkers are investigating the production of extrolites (2019) and volatile organic compounds (VOCs; unpublished) produced by non-aflatoxigenic *A. flavus* strains as potential mechanisms. Chemosensing of these compounds by aflatoxigenic strains may regulate production of toxic secondary metabolites. Findings suggest there are uncharacterized extrolites, and known VOCs unique to non-aflatoxigenic *A. flavus*, that greatly reduce aflatoxin and CPA levels in toxigenic strains. No physical contact was permitted in their experiments to rule out thigmoregulation. Growth (colony diameter and weight) was measured for the extrolite study to assess the mechanism of competitive exclusion or displacement. The observed toxin reductions did not correlate with notable absence of, or even reduction in, growth of the toxigenic strains tested. In the VOC experiments, two compounds significantly reduced aflatoxin production, while completely inhibiting CPA production. More work needs to be conducted to determine if all non-aflatoxigenic strains (including those used as biocontrol agents) produce the same extrolites and VOCs with the capacity to inhibit aflatoxin production. There may be multiple mechanisms of biocontrol, and by taking more time to explore them we may further improve the *A. flavus* biocontrol strategy.

If we learn all the ways a biocontrol strain effects control over aflatoxigenic fungi, we may better wield that knowledge to our advantage. And what if that mechanism differs among non-aflatoxigenic strains? Some non-aflatoxigenic strains may be better than others. One may be more aggressive as a colonizer of host tissue, and another may produce a highly inhibitive compound (extrolite and/or VOC) that turns off aflatoxin production. Maybe there is a different mechanism… or multiple mechanisms acting in concert. It is no longer about "Here is a non-aflatoxigenic strain, let's use it as biocontrol". The time has come to think about how to improve current methods of biocontrol, so a practical consideration should definitely be how a candidate strain works.

What alternative strategies can supplement biocontrol?

As mentioned earlier, there is no "silver bullet" when it comes to controlling aflatoxigenic fungi or preventing aflatoxin contamination, so every aflatoxin mitigation strategy we have at our disposal today cannot yet offer complete inhibition of aflatoxigenic fungi from field to table. A practical consideration to address this is utilizing more than one strategy in conjunction with application of *A. flavus* biocontrol (Cary et al. 2011). On the pre-harvest front, we could use a modeling system to better predict possible aflatoxin outbreaks and use a targeted approach (instead of a blanket approach) to biocontrol applications. We could use *A. flavus* biocontrol strains with aflatoxin-resistant varieties of a target crop such as maize. Perhaps a combination of both modeling and host resistance would allow the biocontrol strain to achieve total prevention of pre-harvest aflatoxin contamination. Obtaining resistance in a crop requires finding markers associated with natural resistance, which can be difficult (Cary et al. 2011), so efforts to build resistant germplasm for other susceptible crops would be necessary. And it may take considerable effort to convince consumers that plant breeding or transgenic crops to boost host resistance are not going to result in food that alters their fragile human DNA or feed that makes animals become zombies. On the post-harvest front, storage practices that ensure a lack of growth and contamination by aflatoxigenic fungi should also prevent growth and proliferation of non-aflatoxigenic fungi, but in situations where growth conditions become favorable for fungi there could be sprays or fumigants of aflatoxin-degrading or inhibitive compounds, naturally found in non-aflatoxigenic *A. flavus* strains, that quell any aflatoxin being produced. This mimicry ensures that the efficacy of the biocontrol strain is maintained even when the strain itself may be unable to grow.

Concluding remarks

It cannot be stressed enough that the advantages of using non-aflatoxigenic *A. flavus* strains as biocontrol far outweigh the disadvantages (if there are any). The purpose of this document is not to deride or decelerate such an effective aflatoxin mitigation strategy, but to stress potential issues relating to biocontrol that can be easily addressed and help ensure its continued success. *A. flavus* researchers should constantly explore potential hindrances to biocontrol efficacy. The practical considerations highlighted in this critical review are intended as seeds of change that will hopefully germinate in the minds of scientists and bring about an evolution in biocontrol research… even if not reported. We should explore the less potent mycotoxins these fungi can produce and test their additive or synergistic effects, and in the interim seek strains that produce as few toxic secondary metabolites as possible. Relatedly, we should be cautious of how we describe these strains to the General Public since

there is likely no such thing as atoxigenicity in filamentous fungi. We should expect all *A. flavus* strains (including biocontrol) to be sexual until proven otherwise. Only by acknowledging this potential, and addressing it through experimentation and molecular investigation, can we ensure a biocontrol strain's reproductive and genetic stability. We cannot assume all non-aflatoxigenic *A. flavus* strains use a single mechanism to inhibit aflatoxin production in competing strains/species. If we determine which mechanisms are possible, we can better screen for candidate strains that offer enhanced control. Lastly, we should continue to research and implement supplemental strategies that enhance efficacy of *A. flavus* biocontrol strains. These considerations are how *A. flavus* and aflatoxin research will evolve. They should not be regarded as doom-and-gloom or as a means to thwart *A. flavus* biocontrol. The stakeholders that benefit from our science do not understand the nuances of fungi, nor the potential for toxic compounds to remain on their food and feed products. They take things at face value, and it is our duty as researchers to offer them a complete story. If there is potential to improve, then we should acknowledge this and work to develop insurmountable biocontrol strains. Current biocontrol strains are helping for now, and we should continue to improve upon them with something better given what we know about the potential for sexual recombination. If the ways to enhance the effectiveness of biocontrol formulations involve seemingly tedious labor, then for the economic good and health of our global community we should labor until every possible issue has a solution that is ready to be implemented.

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