World Mycotoxin Journal, 2015; 8 (2): 225-233
SPECIAL ISSUE: *Aflatoxins in maize and other crops*

Challenges facing the biological control strategy for eliminating aflatoxin contamination

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Received: 14 March 2014 / Accepted: 23 April 2014

REVIEW ARTICLE

Abstract

Competition with *Aspergillus flavus* isolates incapable of aflatoxin production is currently the most widely used biocontrol method for reducing aflatoxin contamination in maize and cottonseed where aflatoxin contamination is a persistent problem for human and animal health. The method involves spreading non-aflatoxigenic *A. flavus* spores onto the field prior to harvest. How competition works is not fully understood. Current theories suggest that atoxigenic *A. flavus* either simply displaces aflatoxin-producing isolates or that competition is an active inhibition process that occurs when the fungi occupy the same locus on the plant. In this paper we describe several challenges that the biocontrol strategy should address before this practice is introduced worldwide. These include the need to better understand the diversity of *A. flavus* populations in the agricultural soil, the effects of climate change on both this diversity and on plant susceptibility, the ability of the introduced biocontrol strain to outcross with existing aflatoxin-producing *A. flavus*, the adaptation of certain *A. flavus* isolates for predominant growth on the plant rather than in the soil, the difficulty in timing the application or controlling the stability of the inoculum, the effect of the introduction of the biocontrol strain on the soil microenvironment, the potential damage to the plant from the introduced strain, and the overall need to better understand the entire *A. flavus* toxin burden, beyond that of aflatoxin, that may result from *A. flavus* contamination. In addition, the cost/benefit ratio for the biocontrol method should be considered in comparing this method to other methods for reducing food and feed contamination with aflatoxins.

Keywords: *Aspergillus flavus* S and L strains, non-aflatoxigenic, sexual recombination, maize, cottonseed

1. Introduction

Aspergillus flavus is the most common species associated with aflatoxin contamination of agricultural crops (Cotty, 1997; Cotty *et al*., 1994). *A. flavus* is found in temperate and tropical regions in soil and, in agricultural areas, on maize, cotton, tree and ground nuts. Field populations of *A. flavus* are highly diverse and their stability in the soil and on the plant is not well understood. An atoxigenic relative of *A. flavus*, *Aspergillus oryzae*, is widely used in soybean and rice fermentation. It is now increasingly clear that *A. oryzae* is not a separate species, but actually is only one of many examples of atoxigenic variants of *A. flavus* (Chang *et al*., 2006; Geiser *et al.*, 2000). Other aflatoxin-producing fungi have been implicated in contamination of agricultural commodities, such as *Aspergillus parasiticus* which has been associated with contaminations of peanuts in the United States (Horn, 2005), Argentina (Vaamonde *et al.*, 2003), and west Africa (Ismail, 2001); but generally, the predominant contaminating organism is *A. flavus* (Cotty *et al.*, 1994). *A. flavus* appears to be more invasive and out-competes *A. parasiticus* when both species are together in the soil. Another aflatoxigenic species known as *Aspergillus nomius* is more rarely found in the soil, and usually is not associated with agricultural contamination episodes (Bhatnagar *et al.*, 2001; Cardwell and Cotty, 2002; Cotty *et al.*, 1994). The original taxonomic identification of *A. nomius* was first made in 1987 (Kurtzman *et al.*, 1987). Recently, there are several reports that *A. nomius* may be a contaminant of Brazil nuts (Goncalves *et al.*, 2012; Olsen *et al.*, 2008). We found that an isolate from Brazil nut originally identified as *A. nomius* actually was an isolate of *Aspergillus*

bombycis. Morphological similarities among these fungi mean that misidentification of the contaminating organism, in some cases, is possible. For example, a group of aflatoxin B- and G-producing isolates, found to be common in Thai soil, resemble *A. flavus*, but have been conclusively identified as a new clade of *A. nomius* (Ehrlich *et al.*, 2007a) and isolates from west Africa identified as B- and G-producing *A. flavus* (Cotty and Cardwell, 1999) are actually a separate *Aspergillus* species that more closely resembles *A. parasiticus* (Varga *et al.*, 2011).

Currently, the most promising strategy being utilised to reduce pre-harvest contamination of cereal grains, ground and tree nuts with aflatoxin is to introduce non-aflatoxin-producing (biocontrol) *A. flavus* into the crop environment to compete with the naturally occurring aflatoxin-producing strains present in the soil (Cotty and Bayman, 1993; Horn and Dorner, 2002; Horn *et al.*, 2000). This technology is being utilised in cotton- and maize-growing regions in the southern United States (Bock *et al.*, 2004; Jaime-Garcia and Cotty, 2013; Mehl and Cotty, 2009) as well as in regions of Kenya where severe outbreaks of aflatoxin poisoning have occurred in recent years (Atehnkeng *et al.*, 2008; Hell *et al.*, 2008; Ogunbayo *et al.*, 2013). There are several reports that application of biocontrol *A. flavus* is able to reduce aflatoxin levels in treated crops by greater than 80% (Abbas *et al.*, 2012; Alaniz Zanon *et al.*, 2013; Cotty and Bhatnagar, 1994; Dorner, 2009; Mehl and Cotty, 2010). In this paper, we outline what is currently known about the nature of the competition and discuss potential questions that should be addressed before the biocontrol strategy is adopted as the most effective approach for elimination of pre-harvest aflatoxin contamination.

2. Diversity in *Aspergillus flavus* and its role in aflatoxin production

A. flavus is a diverse assemblage of strains which include aflatoxigenic and non-aflatoxigenic strains, sclerotial type variants, strains with variability in responses to light, strains residing in multiple vegetative compatibility groups, and strains with variable ability to colonise living plant tissue. Also, *A. flavus* is a heterothallic species where individual isolates possess functional idiomorphs of only one mating type locus (Moore *et al.*, 2013). As a predominantly saprophytic fungus, *A. flavus* resides in the soil, but as an opportunist it is readily able to colonise most environments whenever there is a rich source of carbon and nitrogen. *A. flavus*'s diversity, therefore, appears to be an evolutionary response to its cosmopolitan distribution. Its main mode of reproduction is by asexual conidial sporulation. *A. flavus* forms sclerotia which are hardened masses of desiccated and melanised mycelia that are able to survive adverse environmental and nutritional conditions. They are also the body known as stromata in which sexual reproduction may occur when they are derived from pairings of opposite mating type strains.

Aspergillus flavus soil populations contain isolates from two morphologically distinct sclerotial size variants, termed the L-strain (also called *A. flavus* Group IB (Geiser *et al.*, 2000)) for isolates with average sclerotial size greater than 400 µm, and the S-strain (Group IA) for isolates with sclerotial size less that 400 µm (Cotty, 1997). Both S- and L-strains of *A. flavus* are found globally in maize-growing regions. When incubated in darkness on typical laboratory growth media, S-strain isolates produce higher levels of aflatoxins, more abundant sclerotia, and fewer conidia. Atoxigenic S-strain isolates are very rarely found in natural environments (Orum *et al.*, 1997). Differences in sclerotial morphology correlate with the differences between S- and L-strain *A. flavus* in the size of a deletion in the *norB-cypA* region of the aflatoxin gene cluster (Ehrlich *et al.*, 2004). *A. flavus* lacks the ability to produce G-aflatoxins due to this gap in the cluster because it includes an essential cytochrome P450-encoding gene, *cypA*. The size of the deletion that causes loss of a portion of *cypA* is 1.5 kb for S-strain isolates and 0.8 kb for L-strain isolates. A recent study found another *A. flavus* variant in Africa with a 2.2 kb gap in the *norB-cypA* region (Probst *et al.*, 2013).

Soil populations of *A. flavus* are also typically composed of isolates from hundreds of different vegetative compatibility groups. Although frequent genetic exchange among these groups has not been observed, evidence of historical recombination in *A. flavus* populations has been observed (Moore *et al.*, 2009). Because the 1.5 kb *cypA/norB* deletion in S-strain isolates is identical to the deletion in *A. oryzae* isolates which possess most of the aflatoxin cluster, *A. oryzae* isolates probably descended from a common ancestor that had the S-strain-type deletion (Chang *et al.*, 2005). Many L-strain isolates that lack the ability to produce aflatoxins also have an identical 1.5 kb *norB-cypA* deletion. When this gap size is included in a phylogenetic analysis of *A. flavus* that also includes the *omtA* gene sequence (Geiser *et al.*, 2000), separate clades were revealed that included members of aflatoxin-producing S-strain isolates, L-strain isolates incapable of aflatoxin production and *A. oryzae*, and L-strain isolates capable of AF production (Figure 1). From this data we concluded that the L-strain is ancestral to the S-strain and to both *A. oryzae* and atoxigenic L-strain isolates (Chang *et al.*, 2006); particularly partial-cluster *A. flavus* which are reported to be experiencing gene loss on a recent time scale (Moore *et al.,* 2009).

On average, 30% of *A. flavus* soil isolates sampled in Arizona belong to the S-strain group (Cotty, 1997; Orum *et al.*, 1997). Because S-strain isolates consistently produce more aflatoxin than L-strain isolates, and because aflatoxin production in this strain is not as strongly affected by nitrogen source, the concentration of S-strain isolates in the

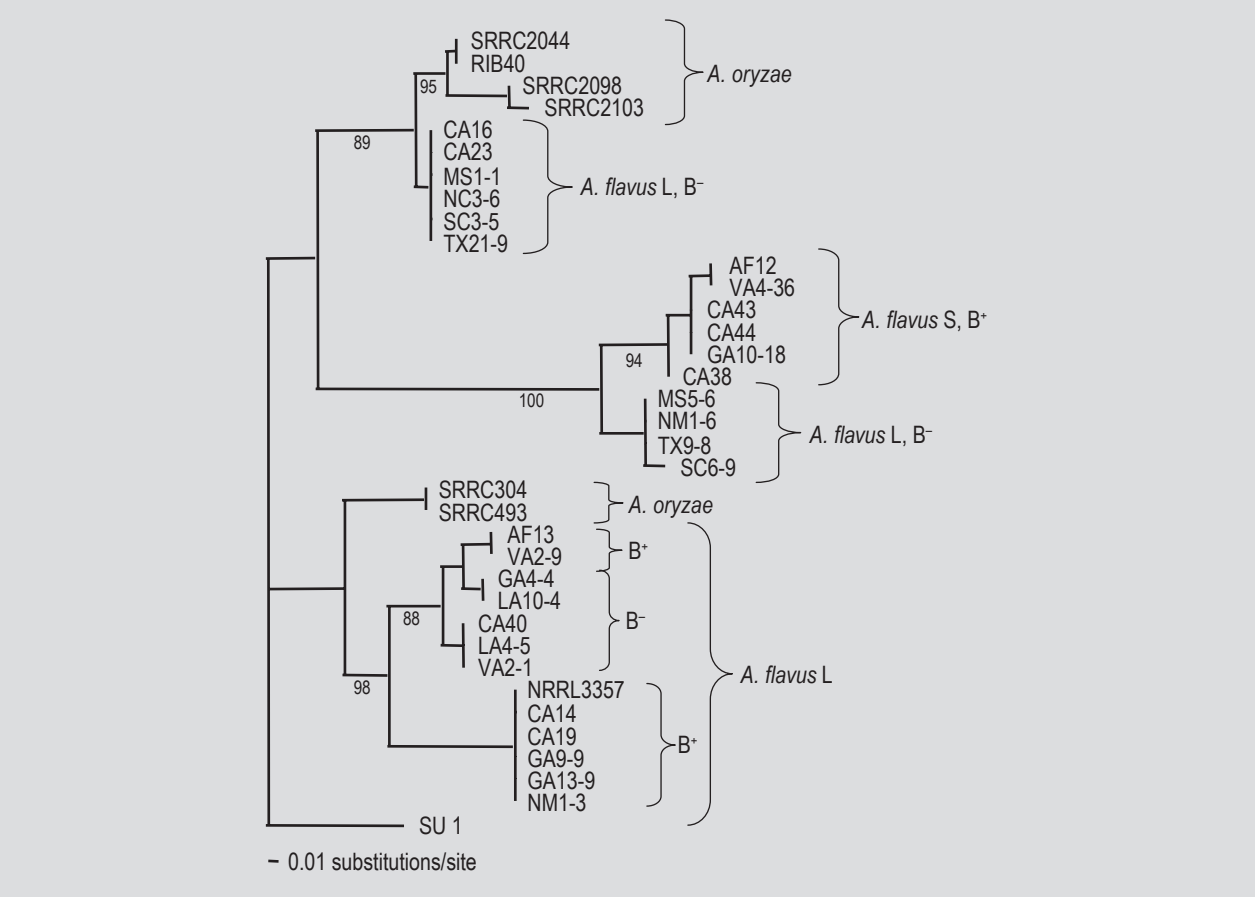

Figure 1. Cladogram illustrating the diversity of *Aspergillus flavus* isolates selected mainly from several different growing areas in the USA. RIB40 is the Japanese *Aspergillus oryzae* strain used for soy and rice fermentations (Ehrlich, 2014). Note that isolates of *A. flavus* with S-strain sclerotia incapable of aflatoxin production are not found, but that a branch of the S-strain clade contains L-strain isolates incapable of aflatoxin production. Isolates from this clade have proven to be the best candidates for aflatoxin biocontrol.

soil appears to be better correlated with major outbreaks of aflatoxin contamination in cotton-growing areas in Arizona and Texas (Jaime-Garcia and Cotty, 2006; Orum *et al.*, 1997). The timing of application was found to be important for effective biocontrol and is crop dependent (Jaime-Garcia and Cotty, 2009, 2013). Furthermore, up to 40% of the L-strain soil isolates of *A. flavus* found in Arizona and other regions of the USA (Horn and Dorner, 1999) were incapable of producing aflatoxins, while S-strain isolates rarely were atoxigenic (Cotty *et al.*, 1994). Interestingly, the isolate, AF36, chosen for cottonseed and maize protection because of its proven ability to compete well with naturally occurring soil isolates, has the S-strain type *norB-cypA* deletion (Chang *et al.*, 2012) even though it is considered to be an L-strain isolate. In summary, the diversity of *A. flavus* represents a problem for developing effective biocontrol *A. flavus* for aflatoxin remediation, but isolates that possess traits of both *A. flavus* S- and L-strains appear to be most suited for the task.

3. Population dynamics of *Aspergillus flavus* in agricultural environments

Recently, *A. flavus*, and many other presumed asexual *Aspergilli*, have been found to be capable of sexual reproduction when grown in the dark under nutrient-deprived conditions (Horn *et al.*, 2009). As a heterothallic fungus, *A. flavus* individuals exist as one of two mating types, *Mat1*-1 and *Mat1*-2 (Ramirez-Prado *et al.*, 2008). Early evidence from genetic analyses suggested that *A. flavus* populations are capable of recombination (Geiser *et al.*, 1998). In these early studies it was thought that *A. flavus* in different vegetative compatibility groups (VCG) would not be able to outcross (Ehrlich *et al.*, 2007b), but VCG is not a barrier to sexual recombination (Horn *et al.*, 2009; Moore *et al.*, 2013). Recombination can occur during sexual crosses between individuals with different mating type loci or within mycelia, conidia or sclerotia if they harbour heterokaryotic nuclei (Olarte *et al.*, 2013). The frequency of mating-type genes in the population is correlated with recombination in the aflatoxin gene cluster (Moore *et*

al., 2013). Recombination has been detected between aflatoxigenic and non-aflatoxigenic *A. flavus* with some of the offspring regaining the ability to produce aflatoxins (Olarte *et al.*, 2012). Clearly, such recombination is a source of diversity within *A. flavus*. Because of this ability to recombine, the frequency of such events in agricultural environments where atoxigenic biocontrol *A. flavus* have been introduced needs to be assessed. Another study found that, while soil populations in agricultural environments had equal populations of fungi with both mating types, the population obtained from the plant (maize) was skewed to overrepresent *Mat1*-2 fungi (Sweany *et al.*, 2011). A recent study found that sclerotia formed on maize are incipient stromata, which when aged on soil produce asci and ascospores indicating sexual reproduction (Horn *et al.*, 2013). These recent studies show that a combination of population genetic processes, asexual/sexual reproduction, coupled with ecological factors, influence recombination.

All of the commonly used non-aflatoxigenic strains of *A. flavus* have the *Mat1*-2 phenotype and a S-strain type *norB-cypA* intergenic region (Chang *et al.*, 2012). This observation suggests that the ability to prevent aflatoxin by these isolates is an active process and not simply displacement. Recent work in *Aspergillus nidulans* has shown that secondary metabolite production and development is controlled, in part, by metabolites at the cell surface (Lo *et al.*, 2012). Although not proven, it is possible that these signalling molecules, or protein factors whose biosynthesis is controlled by the mating-type locus genes, actively prevent aflatoxin production in strains with the opposite mating type. It is certainly reasonable to suggest that biocontrol by non-aflatoxigenic strains is not simply a displacement process, because, if that were the case, all non-aflatoxigenic *Aspergilli*, including *A. oryzae*, would be effective biocontrol agents and that has not proven to be the case. Differential abilities of non-aflatoxigenic and aflatoxin-producing strains to utilise available plant nutrients have not been demonstrated (Mohale *et al.*, 2013) and therefore, this explanation as a mechanism to explain the decreased aflatoxin production by biocontrol competition is unlikely.

It has been shown that there is a frequent loss of aflatoxin-producing ability in *A. flavus* isolates from agricultural soil (Horn, 2007). This could be a consequence of adaptation to a carbon-rich environment that makes the aflatoxin cluster less genetically stable because of a loss in selective pressure to retain the gene cluster. The ability to produce aflatoxins (and other mycotoxins) may give the fungi a long-term advantage over non-aflatoxigenic biocontrol strains for survival in the soil, but in agricultural environments this adaptive pressure may not be required and therefore, not involved in niche selection (Cary and Ehrlich, 2006). Larger effective population sizes tend to increase mean population mutation and recombination rates (Hartl and Clark 1997), further driving the evolution of new VCGs, some of which have lost aflatoxin-producing ability due to mutations within the biosynthetic cluster or to large chromosomal deletions resulting in losses of subtelomeric genes. Since the aflatoxin and cyclopiazonic acid (CPA) clusters reside near the telomere of chromosome 3 in *A. flavus*, such mutations result in a high frequency of loss of aflatoxin and CPA-producing ability (Chang *et al.*, 2005). The detection of conserved sequence breakpoints in both complete and partial aflatoxin gene clusters indicates that recombination has played a large role in cluster disassembly, and multilocus coalescent analyses involving partial cluster strains shows evidence of lineage-specific gene loss in *A. flavus* (Moore *et al.,* 2009). In summary, the diversity of *A. flavus* may be a function of its ability to outcross by sexual recombination under special conditions in the soil. Such outcrossing could potentially cause non-aflatoxigenic strains to gain the ability to make aflatoxin.

4. Other secondary metabolite gene clusters in *Aspergillus flavus*

A. flavus is able to produce other toxic secondary metabolites (Figure 2) which, in addition to aflatoxins, could be of concern for evaluating the safety of the biocontrol strategy. These metabolites could exert their own toxic effects or act synergistically with aflatoxins. The reported toxic effects on humans of ingestion of *A. flavus*-contaminated maize was growth retardation, immune suppression, and liver damage, the latter being associated with hepatitis B infection (Palliyaguru and Wu, 2013). It is possible that some of these toxic effects are, in part, exacerbated by the presence of high levels of aflatrem (and/or its precursor indole diterpenes), aflavinines, or CPA. Metabolites such as aflatrem (Gallagher and Wilson, 1979), paxillines, paspalicines (Nicholson *et al.*, 2009), and aflavinines could act as tremorgens and could cause damage to muscle and nerve function (Selala *et al.*, 1989), particularly during childhood development. Other toxins commonly produced by *A. flavus* are CPA and pseurotin (Varga *et al.*, 2012). CPA inhibits the calcium pump by blocking the cellular calcium access channel and immobilising a subset of four transmembrane helices of ATPase (Moncoq *et al.*, 2007). Besides the specific inhibition of ATPase activity which mainly affects muscle function, CPA can induce various pathological lesions in test animals (Burdock and Flamm, 2000). Another frequently produced metabolite of *A. flavus* includes the Substance P neurotransmitter antagonist, ditryptophenaline (Overman and Paone, 2001). None of these metabolites is currently regulated as a food or feed contaminant. We have recently determined that these metabolites in the laboratory are produced in greater quantities in S-strain *A. flavus* than in L-strain and are produced by some of the non-aflatoxigenic competitor strains (Ehrlich and Mack, unpublished observations). The S-strain was the predominant *Aspergillus* strain in the regions in Kenya and Nigeria where recently severe

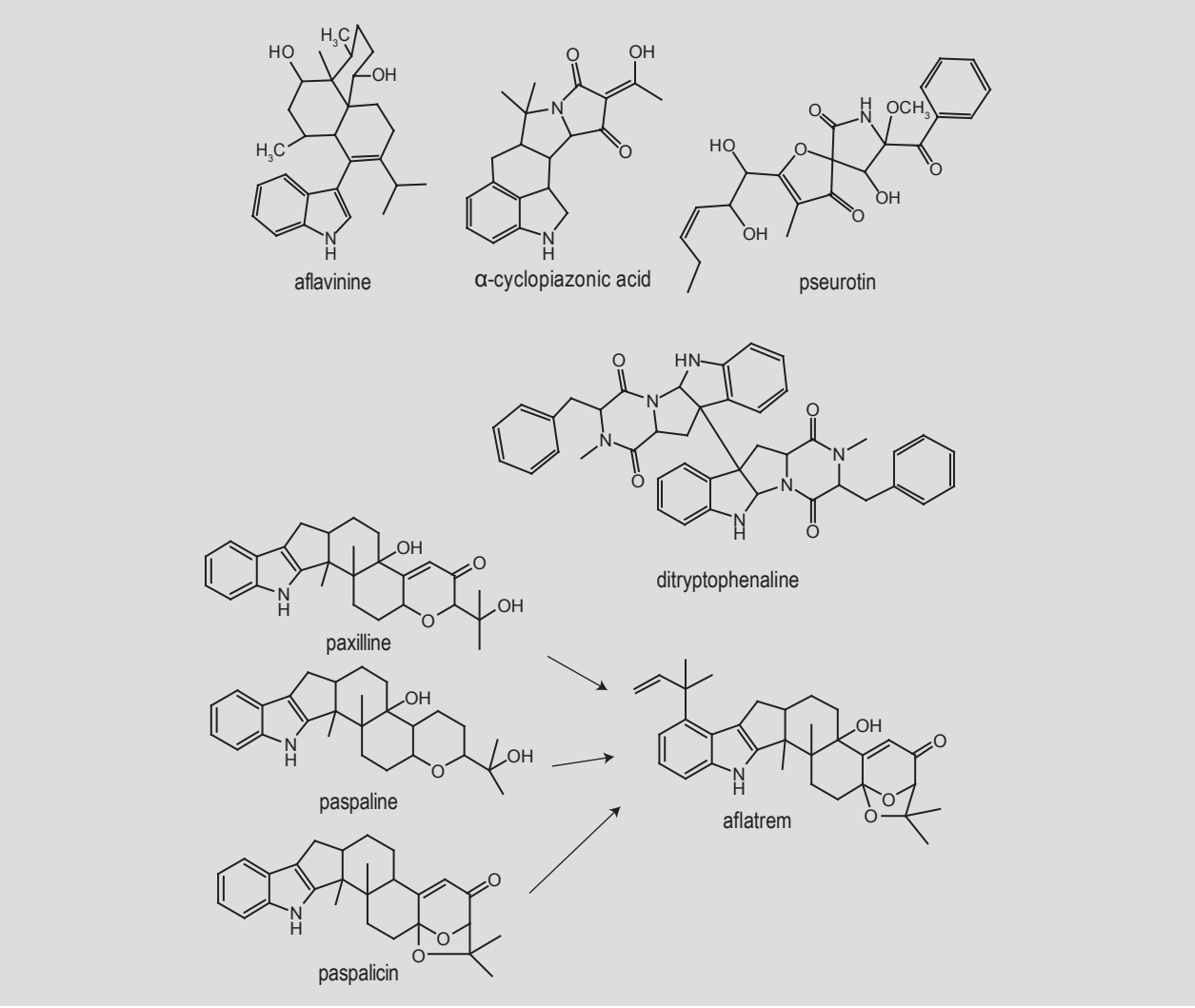

Figure 2. Some known non-aflatoxin metabolites from *Aspergillus flavus* (Ehrlich, 2004).

outbreaks of aflatoxicosis have been reported (Donner *et al.*, 2009; Mehl and Cotty, 2010; Probst *et al.*, 2010).

Maize, unlike groundnuts or cottonseed, can be simultaneously contaminated with both *A. flavus* and fumonisin-producing *Fusaria* (Probst *et al.*, 2013; Shephard *et al.*, 2013; Shirima *et al.*, 2013). Fumonisin is a powerful cancer promoter and its presence could be another factor causing increased cancer risk from ingestion of aflatoxin-contaminated maize. Because of the likely presence of other mycotoxins in contaminated maize, some effort should be made to evaluate their possible roles on the health effects of *A. flavus* ingestion. Presumably, biocontrol fungi, introduced to reduce the aflatoxin burden, will still be capable of producing these other mycotoxins.

5. Climate change and biocontrol

Warming of the earth over the last 100 years has increased daily high temperatures in the mid-west and northern maize-growing regions of the USA and Canada. The resulting temperatures in these regions may begin to resemble those in the southern United States where aflatoxin contamination of maize is a chronic problem. With the increase in temperature will most likely also be an increase in atmospheric CO_2 levels and in frequency of drought conditions. These changes could have a profound effect on crop growth. The potential effects of climate change on mycotoxin production have been recently reviewed (Magan *et al.*, 2011; Medina *et al.*, 2015). Aflatoxin contamination events are more prevalent during times of high heat and drought, conditions which may stress the host plant, possibly weakening plant defence mechanisms, thereby, facilitating *A. flavus* infection (Cotty and Jaime-Garcia, 2007; Hill *et al.*, 1983; Sanders *et al.*, 1984). In addition, increased water stress has been correlated with increased

aflatoxin production. Stress to the fungus could also lead to increased levels of sexual reproduction as discussed above as well as induction of transcriptional activators of development and secondary metabolism (Reverberi *et al.*, 2013). An illustration of the effects of climate change on a shift in mycotoxin production was seen in northern Italy during a particularly hot and dry period. Whereas *Fusarium* contamination normally was a problem, during these dry years *A. flavus* contamination became a more important problem because of *A. flavus* being better able to tolerate higher temperatures and drought (Mohale *et al.*, 2013). Currently, incidences of aflatoxin outbreaks are limited to tropical and temperate areas around the world (Cotty and Jaime-Garcia, 2007; Paterson and Lima, 2012). However, if current scientific reports are accurate, the average global surface temperature may increase by 4 °C by 2100. If such a temperature shift occurs aflatoxin contamination outbreaks could encompass more of the major maize-growing agricultural regions of the USA and Canada. Therefore, it is imperative that research continues to seek aflatoxin control measures that are not subject to such shifts in climate (Wu *et al.*, 2011).

6. Considerations of cost-effectiveness of atoxigenic *Aspergillus flavus* for biocontrol

The costs of the non-aflatoxigenic *A. flavus* biocontrol strategy for aflatoxin remediation and the potential need for seasonal reapplication must be considered in weighing the use of this method as a means of reducing aflatoxin contamination of food and feed. Some early cost estimates have been made (Cardwell *et al.*, 2001; Robens and Cardwell, 2005) and more complete cost analysis has recently been offered (Wu and Khlangwiset, 2010; Wu *et al.*, 2013). Where aflatoxin contamination is chronic, as in cottonseed in Arizona, the risk can more readily be estimated, but in maize where the degree of contamination is more difficult to estimate, unnecessary biocontrol *A. flavus* treatment could be an unacceptable additional cost for the grower.

It has been estimated that biocontrol using atoxigenic *A. flavus* for remediation of aflatoxin contamination of cottonseed and ground nuts in the USA should be cost-effective for most years because aflatoxin contamination is a chronic occurrence in these crops (Wu *et al.*, 2008). The estimate of cost-effectiveness for use of biocontrol in developed countries is based on market factors and is largely based on the consideration that the contaminated commodity may either not be marketable or be of reduced value. For poorer countries, such as those in sub-Saharan Africa, consideration is made additionally for the estimated role of aflatoxin ingestion on causing human disability, the subsequent loss of productivity of the affected worker, and attendant heath care costs. In this latter case, the benefits of crop treatment could outweigh the cost for the treatment (Wu, 2015; Wu and Khlangwiset, 2010; Wu *et al.*, 2013). The difficulty in these cost analyses is that the cost for production of the biocontrol *A. flavus* and for the treatment of fields is now mostly subsidised by government or private foundations and the true costs may be harder to estimate.

Also, the question of the effectiveness of the treatments in reducing aflatoxin levels to those safe for human and animal ingestion has not yet been fully addressed and appear to be quite variable. Furthermore, the levels of aflatoxin contamination which lead to the most severe disability and death result from post-harvest contamination. It is possible that remediation in the field may not prevent such exposure. However, some unpublished evidence suggests that preharvest remediation of aflatoxin by introduction of biocontrol *A. flavus* is able to reduce levels of aflatoxin even in poorly stored maize (unpublished data from www.iita.org). Other methods for reducing aflatoxin contamination, for example, by treatment with bacteria or yeast may be more cost-effective compared to treatment with atoxigenic *Aspergillus* and would be without the risk of exposure to other mycotoxins produced by the fungi (Armando *et al.*, 2012; Hua *et al.*, 2014; Niknejad *et al.*, 2012). In summary, evaluations of cost-effectiveness are complicated by the need to determine the true cost of crop treatment, the ability of the treatment to reduce aflatoxin levels to levels low enough for commerce and human exposure, and the availability of alternative methods or procedures for biocontrol.

7. Conclusion

In its current usage the biocontrol strategy is expected to be able to reduce pre-harvest aflatoxin in cottonseed and maize by as much as 80%. Further work is needed to address the mechanism of biocontrol and its long-term benefits for food and feed safety. The ultimate goal for using non-aflatoxigenic *A. flavus* as a biocontrol agent should be for long-term crop protection. Current strategies utilise a program of annual re-application, but more research is needed to determine the stability of the biocontrol strains and if annual re-application is necessary. Even a low rate of recombination for aflatoxin-producing fungi could be important for future food safety. We suggest in this review that there exist multiple challenges to the biocontrol strategy for prevention of aflatoxin contamination. These challenges result from the inherent diversity of *A. flavus* populations and the now proven ability of *A. flavus* to outcross with aflatoxin-producing isolates in the field. Alterations in plant susceptibility due to climate change could affect contamination levels. Also, with use of the biocontrol *A. flavus*, care must be taken to prevent undue crop damage or damage to the soil microflora. The current strategy should be considered in light of these concerns, especially because introduction of biocontrol *A. flavus* has not consistently been shown to be successful in consistently reducing aflatoxin contamination levels to those needed

for commerce, even when applied under the most optimal of situations (Dorner, 2009).

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