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Review

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Biological Control of Aflatoxin Contamination in US Crops and the Use of Bioplastic Formulations of *Aspergillus flavus* Biocontrol Strains to Optimize Application Strategies

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ABSTRACT: Aflatoxin contamination has a major economic impact on crop production in southern USA. Reduction of aflatoxin contamination in harvested crops has been achieved by applying non-aflatoxigenic biocontrol *Aspergillus flavus* strains that can out-compete wild aflatoxigenic *A. flavus*, reducing their numbers at the site of application. Currently, the standard method for applying biocontrol *A. flavus* strains to soil is using a nutrient-supplying carrier (e.g., pearled barley for Afla-Guard). Granules of bioplastic (partially acetylated corn starch) have been investigated as an alternative nutritive carrier for biocontrol agents. Bioplastic granules have also been used to prepare a sprayable biocontrol formulation that gives effective reduction of aflatoxin contamination in harvested corn kernels with application of much smaller amounts to leaves later in the growing season. The ultimate goal of biocontrol research is to produce biocontrol systems that can be applied to crops only when long-range weather forecasting indicates they will be needed.

KEY WORDS: *Aflatoxins, Aspergillus flavus, biocontrol, maize, peanuts, cottonseed, tree nuts*.

INTRODUCTION

Aflatoxin **[1]** contamination is a primary determinant of crop quality and hence value in corn (maize, *Zea mays* L.) and other crops.[1] Aflatoxin contamination is a particular concern in the southern tier of the US, affecting peanuts (groundnuts) in the east, corn (maize) across the south, cottonseed in Arizona and Texas, and tree nuts in California. A major factor in the cost of aflatoxin contamination to the producer are government regulations which limit crop usage based on aflatoxin contamination levels. Specifically, the US Food and Drug Administration has defined action levels for aflatoxin found in foods for direct human consumption, and feed for dairy cattle and immature animals. Aflatoxin levels must be ≤20 μg/kg except in milk, for which aflatoxin M1 must be ≤0.5 μg/kg; in feeds for breeding beef cattle, breeding swine, or mature poultry aflatoxin levels must be ≤100 μg/kg; in feeds for finishing swine aflatoxin levels must be ≤200 μg/kg; in feeds for finishing beef cattle, aflatoxin levels must be ≤300 μg/kg; and grain with aflatoxin at >300 μg/kg is suitable only for fermentation to produce ethanol. Additional costs associated with aflatoxins in corn include the cost of assaying every commercial lot for aflatoxins, and the cost of government-funded research conducted at universities and national laboratories to seek ways to reduce aflatoxin contamination.

The major route of infection of corn kernels by *A. flavus* is believed to occur from the soil reservoir, when dust particles carrying *A. flavus* conidia are blown by winds from the soil surface to the silks, which emerge on corn ears during R1, the first recognized stage of the reproductive period.[2] The *A. flavus* conidia germinate on a corn silk, penetrate it, grow down it to reach the kernel with which that silk is associated, where the fungus establishes an infection. Lateral spread of the fungus to adjacent kernels may occur later.

In the US and other developed countries aflatoxin contamination of corn kernels almost always occurs pre-harvest, because techniques have been implemented that effectively prevent post-harvest production of aflatoxins, whereas techniques that effectively prevent pre-harvest production of aflatoxins have proven to be much more difficult to develop. Typically corn kernels are dried to less than 15% moisture content immediately after harvest, often with propane-powered grain dryers, and then the kernels are placed under storage conditions that continuously maintain a moisture level low enough to prevent *A. flavus* growth or aflatoxin production. Government regulatory agencies assume that aflatoxin content in corn can be assayed after harvest and the levels will not rise during storage. In developed countries this assumption is generally valid, but in developing countries, where corn not meant for export is often stored under conditions that are far from ideal, substantial post-harvest aflatoxin production may occur.

A variety of strategies for reducing aflatoxin content in harvested corn have been investigated. Extensive efforts made to breed corn cultivars resistant to *A. flavus* infection or to aflatoxin accumulation in kernels have yet to solve the problem.[3] Use of Bt corn was expected to result in less aflatoxin contamination by reducing vectoring of *A. flavus* by insects eating through the husks to the kernels. However, the results of studies of the effects of Bt on aflatoxin contamination in harvested corn have been inconsistent,[4] suggesting that the types of insects controlled by Bt do not always play a major role as *A. flavus* vectors in corn.

Some success in reducing aflatoxin contamination has been obtained using agronomic cultural methods.[5] For many growing seasons, even in the southern US, aflatoxin contamination of harvested corn is very low. However, when the weather is hot and dry during the kernel filling period, particularly when night temperatures are greater than 68^{o}F, substantial aflatoxin

92 contamination can occur, consistent with heat and drought stressing the plant to the point that
93 resistance to *A. flavus* infection, growth and aflatoxin production is reduced.[6] In the southern US
94 the corn growing season is long enough so that the planting date can be adjusted to allow the
95 kernel filling period to occur when rainfall is traditionally adequate and temperatures moderate
96 in the region.[7]
97
98 **PRINCIPLE OF BIOCONTROL**
99 Biocontrol of mycotoxin contamination in crops has been successful by applying a non-toxigenic
100 strain of the fungus to a convenient ecological niche normally occupied by toxigenic wild type
101 fungus.[8-13] Biocontrol fungal strains do not produce toxins, and must suppress multiplication of
102 wild-type toxin-producing fungi, by out-competing toxigenic strains for ecological niches.
103 Biocontrol fungi must also be readily cultured and stable to storage and application conditions.
104 Traditionally the site of application of biocontrol fungi in the US has been the soil. For a crop
105 such as peanuts, soil applications or seed coatings will always be the method of choice. For
106 other crops, such as corn or tree nuts, applying the biocontrol agent at an ecological niche closer
107 to the actual fungal infection site on foliar plant parts can be considered as a strategy for
108 reducing both the treatment amount and the application lead time. Because the ultimate site at
109 which *A. flavus* infects corn kernels are the silks, they may be the primary application site of a
110 biocontrol agent, assuming that both the aflatoxigenic and biocontrol *A. flavus* strains can
111 compete for infection of corn silks. If competition does not take place on the silks, the
112 biocontrol *A. flavus* strain should be applied to the nearest place from which aflatoxigenic *A.*
113 *flavus* comes to infect silks, probably the leaves. Effective reduction of aflatoxin contamination
114 in harvested corn kernels will occur when the biocontrol *A. flavus* strain takes over that

ecological niche and suppresses the number of wild, aflatoxigenic *A. flavus* conidia propagating there.

Aflatoxin contamination of harvested corn reaches government regulatory action levels only during hot, dry growing seasons. In the southern US, these conditions occur frequently, although not every year, whereas they never occur at the northern end of the corn belt. The ultimate goal of research in the field is to develop formulations and application strategies that allow use of a biocontrol agent only when long-range weather forecasts indicate that hot, dry conditions will occur during the kernel-filling period.

BIOCONTROL OF AFLATOXINS IN US CROPS

Research on the development of methods to use biological control to reduce aflatoxin contamination have focused on cottonseed in Arizona, peanuts in Georgia, corn in Mississippi and other southern states, and tree nuts (pistachio, almond and walnut) in California.

Cotton. Initial studies on the development of biocontrol strains of *A. flavus* for the reduction of aflatoxin levels in harvested crops were carried out on cotton by Cotty in Arizona.[8-10] Aflatoxins are not a problem in cotton fiber production, but additional revenues from the sale of cottonseed oil and cottonseed meal are a necessary part of the economics of cotton production. Aflatoxins can be removed from cottonseed oil with a charcoal filter, but removal from cottonseed meal is much more difficult. Cotty screened *A. flavus* isolates for inability to produce aflatoxins, and he selected a non-aflatoxin producing strain (AF36) for study as a biocontrol agent. He demonstrated that AF36 applied to the soil of cotton fields immediately prior to the first bloom was effective at reducing aflatoxins in harvested cottonseed meal.[9,10] AF36 was applied to the soil in the form of sterilized wheat kernels colonized by AF36. This application

technique resulted in suppression of aflatoxigenic *A. flavus* in treated soil and in domination of the ecological niche by AF36. Substantial suppression of aflatoxigenic *A. flavus* by AF36 persisted in the soil into the following year. Reductions in aflatoxin contamination of the cottonseed meal prepared from the crop ranged from 75-99%. *Aspergillus flavus* AF36, manufactured by the Arizona Cotton Research and Protection Council was initially registered with the Environmental Protection Agency (EPA) for cotton in Arizona in November, 2007. *Aspergillus flavus* AF36 was also registered in February, 2012 with the EPA for use on cotton in Arizona, and three counties in California and Texas.

Unfortunately, while AF36 did not produce aflatoxins, it did produce another mycotoxin, cyclopiazonic acid (CPA) **[2]** in peanuts[11] and in corn,[14] which is a major concern for commercial marketers of biocontrol *A. flavus* strains.[15] Cyclopiazonic acid is a mycotoxin structurally similar to ergot alkaloids that is produced by various *Penicillium* spp. and *Aspergillus* spp. Cyclopiazonic acid has been shown to be under the same regulation as aflatoxin in many isolates of *A. flavus* and *A. parasiticus*.[16] Cyclopiazonic acid, an inhibitor of a calcium pump in mammalian calciosomes, is considerably less toxic than aflatoxins but is still a concern as a feed contaminant for young poultry, which are much more sensitive to it than mammals. Indeed, it has been suggested[17] that the effects on turkeys that led to the discovery of aflatoxins in the 1960s were actually caused by CPA produced with some aflatoxins by the *A. flavus* strain that caused turkey "X" disease.

Peanuts. Peanut pods develop in the soil, where they are in direct contact with *A. flavus* and other mycotoxin-producing fungi. The pods are particularly susceptible to infection by *A. flavus* and *A. parasiticus* when subjected to drought during maturation of the kernels (i.e., late in the growth season). Although irrigation during this period can prevent infection by *Aspergillus* spp.,

it is not available to most growers. Dorner[12] used mutants of *A. parasiticus* blocked in aflatoxin biosynthesis to provide proof that non-aflatoxigenic isolates can displace *A. flavus* and *A. parasiticus* in the soil, and reduce aflatoxin contamination of harvested peanut kernels. A wide variety of *A. flavus* and *A. parasiticus* isolates were screened to identify isolates that did not produce aflatoxins, CPA, *O*-methylsterigmatocystin, versicolorins or any other biosynthetic intermediates. An isolate of *A. flavus*, NRRL 21882, designated Afla-Guard, emerged from a comparison of isolates for their ability to reduce aflatoxins in harvested kernels and ultimately was put forward for marketing as a commercial biocontrol strain. Extensive studies aimed at developing a practical application process were conducted. Among the carriers investigated were rice, pre-gelatinized corn flour granules and pasta bits. The final carrier selected was pearled barley. All carriers tested were effective, but pearled barley had advantages in terms of price and ease of manufacturing. Conidia suspensions were sprayed onto unsterilized pearled barley. Large-scale field trials with Afla-Guard were conducted after its conditional registration in 2004.

Corn. Biocontrol of aflatoxin levels in harvested corn kernels differs from biocontrol in peanuts in several important ways. Most notably, corn kernels are located in an aerial part of the plant, not in the soil. Furthermore, there are various infection mechanisms used by *A. flavus* to colonize developing corn kernels, none of which are similar to the sites in peanuts. Silks appear on developing corn ears in R1 about 65 days after planting of corn, and persist about 12 days, when they darken and dry out. Wind-borne dust particles that carry *A. flavus* conidia can stick to silks, where they germinate, then grow down the silk to the kernel to which the silk is attached, and then infect the kernel. Thus, silks may be the ultimate site at which atoxigenic biocontrol *A. flavus* strains compete with environmental aflatoxigenic *A. flavus* strains.[6] The pool of *A. flavus*

in the environment overwinters in soil and plant debris on the soil surface. Aflatoxigenic *A. flavus* in that pool is assumed to reach corn silks primarily on wind-borne dust particles.

Initial studies on biocontrol of aflatoxin contamination in harvested corn kernels were conducted by Dorner,[13] with the aim of extending the technology developed for peanuts, to corn. Studies began in the 2005 and 2006 growing seasons with the granular preparation containing Afla-Guard applied to soil, and to whorls of the corn plant and as an aqueous suspension of conidia, which was applied to silks of the corn plant four times during silking. In 2005, no significant differences in aflatoxin contamination of harvested kernels were observed between soil, whorl and silk application, but in 2006, whorl application was significantly better than soil or silk application at reducing aflatoxin contamination in harvested kernels. This biocontrol product, Afla-Guard, was registered with the Environmental Protection Agency (EPA) for corn, beginning in the 2009 growing season. The product contains 0.0094% (wt/wt) of Afla-Guard conidia equivalent to 1.2×10^8 cfu. Also commercially available for the biocontrol of aflatoxin in corn, in Texas and Arizona, is *A. flavus* AF36 manufactured by the Arizona Cotton Research and Protection Council. It was initially registered with the EPA for cotton in Arizona in November 2007 and the registration was expanded for the treatment of corn in Texas and Arizona in February, 2012.

Tree Nuts. Research on reducing pre- and post-harvest aflatoxin contamination in tree nuts has focused on pistachio, almonds and walnuts. The mycotoxins of greatest concern in tree nut are aflatoxins and ochratoxins. The primary route of infection is insect vectored, so that insect control is the most important strategy for mycotoxin control in tree nuts.[18] Because antioxidants such as tannins, flavonoids and phenolic acids, markedly inhibit aflatoxin production by *A. flavus* in culture, increasing antioxidant levels in tree nuts has been pursued as a strategy for reducing

aflatoxin contamination in harvested nuts.[19] Post-harvest sorting of nuts by machines that detect the blue-green fluorescence of contaminating aflatoxins was unsuccessful, because aflatoxin levels in tree nuts are so low that the fluorescence of kojic acid obscures it.[20] Studies on biological control of aflatoxin production in tree nuts with non-aflatoxigenic *A. flavus* have used AF36 in pistachio orchards.[21] AF36 on wheat was applied to the soil in June or July over four consecutive years (2008 to 2011). Reductions in aflatoxin contamination levels in harvested pistachios of 20-45% were obtained. *Aspergillus flavus* strain AF36, manufactured by the Arizona Cotton Research and Protection Council, was registered in February, 2012, with the US Environmental Protection Agency (EPA) for use on pistachios in Arizona, California, Texas and New Mexico.

OPTIMIZATION OF AFLATOXIN BIOCONTROL IN CORN (MAIZE) IN MISSISSIPPI AND ITALY

Initial studies on biological control of aflatoxins in corn in Mississippi used Afla-Guard, the *A. flavus* isolate identified for biological control of aflatoxins in peanuts in Georgia. It was applied to corn fields using the same type of carrier, pearled barley, and the same delivery site, the soil.[22, 23] This approach was successful enough to result in EPA registration as Afla-Guard for corn. However, corn is different from peanuts in many ways, so that there was believed to be a reasonable possibility that a corn-associated biocontrol *A. flavus* strain could be more effective than a peanut-associated strain, the reason being that the infection site and the infection mechanism in corn, differs markedly from that in peanuts. In peanuts, *A. flavus* in its natural habitat, directly invades peanuts also located in the soil, if plant defenses are weakened by

drought. Insect and nematode vectoring are alternate routes. Thus, for peanuts, treating the soil with the biocontrol agent is the logical option.

While the major *A. flavus* infection route in corn is via wind-borne dust particles bearing conidia landing on silks during R1,[6] vectoring of *A. flavus* conidia by foliar feeding insects that physically breach the husk represents a significant alternate infection mechanism. Inoculating soil with an *A. flavus* biocontrol strain is effective in reducing dust-borne wild-type aflatoxigenic *A. flavus* spores reaching the silks to the extent that the biocontrol strain out-competes indigenous *A. flavus* and replaces it in the soil reservoir. It is presumably the property of soil competitiveness that makes Afla-Guard effective in controlling aflatoxin contamination in both peanuts and corn. However, corn in principle offers opportunities to improve aflatoxin reduction by applying the biocontrol *A. flavus* strain closer to the silks in distance and time, than is required for application to soil.

Accinelli et al.[24-28] have carried out a series of studies, still ongoing, aimed at improving biocontrol of aflatoxins in corn. These optimization studies have focused on four areas: (a) non-aflatoxigenic *A. flavus* strain selection; (b) inoculum carrier optimization; (c) application site optimization; and (d) application time optimization.

Biocontrol *A. flavus* Strain Optimization. While initial studies on aflatoxin biocontrol in corn were successful using the *A. flavus* biocontrol strain developed for peanuts, NRRL 21882, with the same carrier (inoculated pearled barley) and the same application site (soil) and resulted in an EPA-registered commercial product (Afla-Guard), corn does differ from peanuts in enough ways that there is a good possibility that a corn-specific biocontrol strain might be more effective with respect to efficacy and cost. The only way to determine if a non-aflatoxigenic strain of *A. flavus* selected from corn would be a more effective biocontrol agent on corn than Afla-Guard

252 isolated from peanuts or biocontrol strain *A. flavus* NRRL 18543 (AF36) isolated from cotton,
253 was to select potential biocontrol non-aflatoxigenic strains from corn and compare them head-to-
254 head. Because most isolates of *A. flavus*, particularly from soil do produce aflatoxins, the search
255 for non-aflatoxigenic corn-associated *A. flavus* strains was facilitated by using in culture assay
256 systems to eliminate most aflatoxigenic strains in step 1 of the screening. The two available in
257 culture assays, the Lin and Dianese[29] test (yellow color on the back of colonies that produce
258 aflatoxins) and the Saito and Machida[30] test (red color produced on exposure to ammonia vapor)
259 were both empirical. They were therefore examined and shown to involve detection of the same
260 pigments, which were biosynthetic precursors of aflatoxins.[31] Confirmation of atoxigenicity was
261 accomplished by growing isolates on solid autoclaved grain medium, extraction using standard
262 conditions for aflatoxins and quantitative measurement of any aflatoxins by HPLC-based
263 methods.

264 The resulting corn-associated non-aflatoxigenic biocontrol *A. flavus* strain NRRL 30797
265 (K49) was subjected to head-to-head comparisons with Afla-Guard and AF36 in a series of trials
266 in corn in Mississippi between 2007 and 2009.[24] The results obtained indicated that the corn-
267 associated non-aflatoxigenic strain K49 applied to the soil was not significantly better at
268 reducing aflatoxins and cyclopiazonic acid in harvested corn kernels than Afla-Guard, but both
269 K49 and Afla-Guard were significantly better than AF36.[24]

270 **Fungal Carrier.** For soil application of biocontrol *A. flavus* strains to corn in Mississippi,
271 initial studies used the standard pearled barley carrier used in studies on peanuts. Subsequent
272 research has focused extensively on the use of starch-based bioplastic granules for soil
273 application, field monitoring and as aqueous suspensions. Studies have been carried out with a
274 commercial starch-based bioplastic. Most commercial starch-based bioplastics are prepared

from corn starch by acetylation with acetic anhydride and dilute sodium hydroxide. The extent of acetylation is usually relatively low, typically less than 10% acetate, so that most commercial bioplastics retain desirable starch properties including wettability and susceptibility to amylase-catalyzed hydrolysis that allows them to provide a nutrient source for any biocontrol fungus they may be being used for as a carrier. Acetylation also results in increased hydrophobicity, giving a compact product with good physical stability.

Initial studies with granules of commercial starch-based bioplastic as a direct replacement for pearled barley[24] showed that it effectively absorbs *A. flavus* conidia and allows good viability for storage on the shelf for up to six months. In soil, inoculated bioplastic granules persisted in identifiable form for more than two months and supported maximal colonization of native and sterilized soils by biocontrol *A. flavus* strain K49 in 30 days. Similarly, displacement of aflatoxigenic *A. flavus* strain NRRL 30796 by biocontrol *A. flavus* strain K49 in native or sterilized soil was maximal by 30 days.

Bioplastic granules that had been impregnated with biocontrol *A. flavus* strain K49 conidia and dried were field tested in 2009 and 2010 at 15 and 30 kg/ha.[25] The total *A. flavus* density in soil remained near a typical 3.1 $\log_{10}$ cfu/g in untreated plots, but plots treated with 15 and 30 kg/ha of biocontrol *A. flavus* on bioplastic granules experienced modest but significant increases in total (i.e., aflatoxigenic plus non-aflatoxigenic) *A. flavus* over a 4-month period. However, the percent aflatoxigenicity of isolates from treated plots fell steadily over a 4-month period from about 40% to about 10%, whereas the percent aflatoxigenicity of isolates from untreated plots did not change significantly. Soil application of biocontrol *A. flavus* at 15 kg/ha resulted in a 59% reduction in aflatoxin contamination in harvested corn kernels in 2009 and an 80% reduction in 2010, whereas application at 30 kg/ha resulted in an 86% reduction in aflatoxin

298 contamination in 2009 and a 92% reduction in 2010. Bioplastic granules also proved to be
299 useful probes of the *A. flavus* composition in field soil. Bioplastic granules that had not been
300 inoculated remained intact in soil, where they become impregnated with the *A. flavus* that are
301 living in the soil. Total *A. flavus* DNA in bioplastic granule probes did not correlate with
302 aflatoxin contamination in harvested corn kernels. However, when granules used for baiting
303 Aspergilli from kernel samples were incubated in test tubes containing yeast sucrose and then the
304 medium analyzed for aflatoxin concentrations, a significant correlation between the amount of
305 aflatoxin produced by baited fungi and aflatoxin contamination of corn kernels was found.[25]
306 A comparison of the effectiveness of bioplastic granules as a carrier for biocontrol *A. flavus*
307 strain K49 was conducted in 2011 and 2012 in Northern Italy and in Mississippi at 15 and 30
308 kg/ha.[26] The 2012 growing season was sufficiently hot and dry in both countries to provide a
309 good test of the effectiveness of a biocontrol system. In Northern Italy the aflatoxin levels in
310 untreated control plots in 2012 were seven times that in 2011. In 2012 in Northern Italy
311 application of biocontrol strain K49 on bioplastic granules at 15 kg/ha reduced aflatoxin
312 contamination in harvested corn kernels by an average of 67 ± 4.1%, whereas at 30 kg/ha it
313 reduced aflatoxins by an average of 94.8 ± 5.3%. In Mississippi two biocontrol *A. flavus* strains,
314 Afla-Guard and K49 were compared, both at 30 kg/ha, in Bt and non-Bt corn. Both biocontrol
315 *A. flavus* strains were highly effective at reducing aflatoxin contamination in harvested corn
316 kernels, but the corn-derived biocontrol *A. flavus* strain K49 reduced the residual aflatoxin
317 contamination level to about half the level observed with the peanut-derived biocontrol *A. flavus*
318 strain, Afla-Guard. There were no significant differences in biocontrol effectiveness between Bt
319 and non-Bt corn.
320

APPLICATION SITE FOR BIOCONTROL *A. FLAVUS*

Almost all of the early research on biocontrol of aflatoxin contamination in various crops have used a nutrient-rich carrier applied to the soil. In the case of peanuts, soil application is the only reasonable option, because the harvested crop develops in soil, but for other crops that have been subjects of aflatoxin biocontrol research, the harvested crop develops in aerial parts of the plant – the ears in corn, the bolls in cotton and the seed inside a hard shell in tree nuts. In principle, if a biocontrol agent is applied closer to or at its ultimate site of action, it should be possible to apply it later and in smaller amounts. In the case of corn, the ultimate site of interaction between the biocontrol *A. flavus* strain and aflatoxigenic soil-derived *A. flavus* is believed to be the silks, but no one has developed a good way to apply biocontrol *A. flavus* to corn silks. Applying biocontrol *A. flavus* to upper leaf surfaces of corn is closer to the ultimate site of competition, and because total leaf surfaces are inherently smaller than the soil area, a lesser number of biocontrol cfu should be needed. There are potential cost benefits from applying smaller amounts, but the greatest potential benefits would come from reducing the application lead time to less than, or equal to, the time of long-range weather forecasts.

Dorner[13] compared application of biocontrol *A. flavus* strain Afla-Guard in pearled barley (a) to soil at 22.4 kg/ha; (b) to plant whorls at 22.4 kg/ha and (c) as a conidial suspension with no nutrient source sprayed from above, four times during silking in 2005 and 2006. In 2005 weather conditions resulted in low levels of aflatoxin contamination and no significant difference from the control. However, in 2006 aflatoxin contamination of harvested corn was high in untreated control corn and significantly reduced by all biocontrol treatments. Whorl application gave the best results in the first 2006 planting, reducing contamination to about half the level of that remaining after soil application of the same strain in pearled barley. The spraying conditions

used in the study were intermediate in effectiveness between whorl and soil application. In a second planting, only whorl application significantly reduced aflatoxin contamination in harvested corn kernels.

Accinelli et al.[28] developed a sprayable formulation for biocontrol *A. flavus* strains using finely divided pre-gelatinized corn starch-based bioplastic. Acetylation of starch to less than 10% acetate substantially alters the properties of the starch, reducing wettability to some extent, but increasing adherence to cuticle-coated leaf surfaces and still allowing degradation by amylases so that it can still provide nutrients to support growth of a biocontrol strain of *A. flavus* or other biocontrol fungus. Another starch property that is retained is gelatinization by heating in water at 80^{o}C or higher. Gelatinization creates deformable particles that go through a sprayer head better without sacrificing other desirable properties. These small particles can still act as a nutrient source that allows a biocontrol fungus like *A. flavus* strain K49 to produce sufficient conidia to compete with aflatoxigenic *A. flavus* from the soil reservoir. Although the small particles support production of fewer biocontrol *A. flavus* conidia, the production is closer to the site of competition (the silks) than is soil so it was expected to be sufficient.

In Northern Italy in 2012 weather conditions were hot and dry, favoring aflatoxin contamination of harvested corn kernels. A 1% bioplastic-based formulation with *A. flavus* strain K49 as the biocontrol strain, was sprayed on the leaves of corn growing on untreated soil at one sixth the inoculum size normally used for soil application. Application of the sprayable formulation resulted in an average 96.5% reduction in aflatoxin contamination of harvested corn kernels relative to untreated control plots. An additional set of treatment groups had the soil amended with untreated corn field plant material residues. This treatment resulted in slightly higher aflatoxin contamination in kernels harvested from unsprayed controls, but leaf application

of the sprayable biocontrol *A. flavus* formulation resulted in an average 97.1% reduction in aflatoxin contamination of harvested corn kernels relative to unsprayed control plots.[32] Amending the soil with corn field plant material residues inoculated with aflatoxigenic *A. flavus* NRRL 30796 further increased aflatoxin contamination in kernels harvested from unsprayed controls. However, leaf application of the sprayable biocontrol *A. flavus* formulation resulted in an average 96.9% reduction in aflatoxin contamination of harvested corn kernels relative to unsprayed control plots. Examination of corn leaf surfaces after applying the sprayable biocontrol *A. flavus* formulation indicated effective reduction in the percent aflatoxigenicity of indigenous *A. flavus* relative to the unsprayed leaves of control corn plants. In contrast, spraying the biocontrol *A. flavus* formulation on corn leaves had no significant effect on the amounts or percent aflatoxigenicity of *A. flavus* in the soil under the plants.[32]

Weaver et al.[33] evaluated two sprayable formulations of biocontrol *A. flavus* strain Afla-Guard in 2011 and 2012. They found that one water dispersible granule formulation gave an average of 49% reduction in aflatoxins in harvested corn kernels.

APPLICATION TIME

Studies have been conducted to determine the optimal time to apply commercial biocontrol *A. flavus* products, AF36 and Afla-Guard, to the soil to reduce aflatoxin contamination of harvested corn. Mays et al.[34] compared application at V8 (the 8-leaf vegetative stage) to VT (tasseling, the last vegetative stage that occurs 9 to 10 weeks after emergence) and obtained better results with application at V8. Other studies have indicated optimal aflatoxin reduction in harvested corn with Afla-Guard occurs when it is applied in V10 to V12.[35] Detailed studies on

389 the optimal application time for sprayable bioplastic formulations of Afla-Guard and K49 are at
390 the planning stage.
391
392 **OUTLOOK FOR FUTURE PROGRESS**
393 Aflatoxin contamination outbreaks are usually triggered by hot, dry weather conditions.
394 Long-range weather forecasting is expected to improve, particularly as more advanced weather
395 prediction satellites come online. The ultimate goal of biocontrol research is to develop *A. flavus*
396 biocontrol strain formulations and application techniques that allow use of the technology close
397 enough to the kernel filling period that long-range weather forecasting can reliably predict its
398 need. It is hoped that both biocontrol technology and weather prediction will advance to permit
399 such a convergence in the near future.
400 Presently, biocontrol fungus inoculum size, treatment time, number of treatments and site of
401 treatment need to be optimized for sprayable bioplastic-based formulations. The minimum time
402 and formulation conditions required to achieve dominance by the biocontrol *A. flavus* over
403 naturally occurring *A. flavus* on leaves, and the persistence of that dominance should be
404 determined. Studies on fungal DNA accumulated on leaves[28,32] suggest that three weeks is
405 required to achieve optimal dominance of biocontrol *A. flavus* K49 after spraying on leaves, but
406 the generality of this observation under other weather conditions (rainfall and temperature) needs
407 to be determined. It also needs to be determined if application of biocontrol formulations
408 directly to silks in R1, or the use of more effective formulations of biocontrol *A. flavus* can result
409 in reduced aflatoxin levels in harvested kernels.
410 All biocontrol agents currently available commercially are applied to soil every year before it
411 is known whether weather conditions will make treatment necessary. The probability that the

treatment will be needed declines as one progresses north into more temperate regions, where the frequency of aflatoxin contamination outbreaks is low enough that the expense of annual soil treatment cannot be justified. It is in these regions that a biocontrol technology that is applied only when needed will have more favorable cost-benefit considerations and result in wider application of biocontrol and less aflatoxin entering the food and feed supplies.

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REFERENCES

(1) Robens, J.; Cardwell, K. The costs of mycotoxin management to the USA: Management of aflatoxins in the United States. *J. Toxicol. Toxin Rev.* **2003**, *22*, 139-152.

(2) Diener, U. L.; Cole, R. J.; Sanders, T. H.; Payne, G. A.; Lee, L. S.; Klich, M. A. Epidemiology of aflatoxin formation by *Aspergillus flavus*. *Annu. Rev. Phytopathol*. **1987**, *25*, 249–270.

(3) Williams, W. P.; Windham, G. L.; Buckley, P. M. Enhancing maize germplasm with resistance to aflatoxin contamination. *J. Toxicol. Toxin Rev.* **2003**, *22*, 175-193.

(4) Abbas, H. K.; Zablotowicz, R. M.; Weaver, M. A.; Shier, W. T.; Bruns, H. A.; Bellaloui, N.; Accinelli, C.; Abel, C. A. Implications of Bt traits on mycotoxin contamination in maize: overview and recent experimental results in southern United States. *J. Agric. Food Chem*. **2013**, *61*, 11759-11770.

(5) Bruns, H. A. Controlling aflatoxin and fumonisin in maize by crop management. *J. Toxicol. Toxin Rev.* **2003**, *22*, 153-173.

(6) Fountain, J. C.; Scully, B. T.; Ni, X.; Kemerait, R. C.; Lee, R. D.; Chen, Z.-Y.; Guo, B. Environmental influences on maize-*Aspergillus flavus* interactions and aflatoxin production**.** *Front. Microbiol*. **2014**, *5*, 40.

(7) Abbas, H. K.; Shier, W. T.; Cartwright, R. D. Effect of temperature, rainfall and planting date on aflatoxin and fumonisin contamination in commercial Bt and non-Bt corn hybrids in Arkansas. *Phytoprotection* **2007**, *88*, 41-50.

(8) Cotty, P. J. Prevention of aflatoxin contamination with strains of *Aspergillus flavus.* *Phytopathology* **1989**, *79*, 1153.

(9) Cotty, P. J. Virulence and cultural characteristics of two *Aspergillus flavus* strains pathogenic on cotton. *Phytopathology* **1989**, *79*, 808-814.

(10) Cotty, P. J. Effect of atoxigenic strains of *Aspergillus flavus* on aflatoxin contamination of developing cottonseed. *Plant Dis.* **1990**, *74*, 233-235.

(11) Dorner, J. W.; Cole, R. J.; Connick, W. J.; Daigle, D. J.; McGuire, M. R.; Shasha, B. S. Evaluation of biological control formulations to reduce aflatoxin contamination in peanuts. *Biol. Control* **2003**, *26*, 318–324.

458 (12) Dorner, J. W. Development of biocontrol technology to manage aflatoxin contamination
459 in peanuts. *Peanut Sci.* **2009**, *36*, 60-67.
460 (13) Dorner, J. W. Biological control of aflatoxin contamination in corn using a nontoxigenic
461 strain of *Aspergillus flavus*. *J. Food Prot*. **2009**, *72*, 801-804.
462 (14) Abbas, H. K.; Weaver, M. A.; Horn, W. B.; Carbone, I.; Monacell, J. T.; Shier, W. T.
463 Selection of *Aspergillus flavus* isolates for biological control of aflatoxin in corn. *Toxin Rev.*
464 **2011**, *30,* 59-70.
465 (15) King, E. D.; Bassi, A. B.; Ross, D. C.; Druebbisch, B. An industry perspective on the use
466 of "atoxigenic" strain of *Aspergillus flavus* as biological control agents and the significance of
467 cyclopiazonic acid. *Toxin Rev.* **2011**, *30,* 33-41.
468 (16) Chang, P.-K.; Horn, B. W.; Dorner, J. W. Clustered genes involved in cyclopiazonic acid
469 production are next to the aflatoxin biosynthesis gene cluster in *Aspergillus flavus*. *Fungal*
470 *Genet. Biol.* **2009**, *46*, 176-182.
471 (17) Cole, R. J. Etiology of Turkey "X" disease in retrospect: A case for the involvement of
472 cyclopiazonic acid. *Mycotoxin Res*. **1986** *2*, 3-7.
473 (18) Campbell, B. C.; Molyneux, R. J.; Schatzki, T. F. Current research on reducing pre- and
474 post-harvest aflatoxin contamination of U.S. almond, pistachio, and walnut. *J. Toxicol. Toxin*
475 *Rev*. **2003**, *22*, 225-266.
476 (19) Molyneux, R. J.; Mahoney, N.; Kim, J. H.; Campbell, B. C. Mycotoxins in edible tree
477 nuts. *Int. J. Food Microbiol*. **2007**, *119*, 72-78.
478 (20) Farsaie, A.; McClure, W.; Monroe, R. Design and development of an automatic electro
479 optical sorter for removing BGY fluorescent pistachio nuts. *Trans. ASAE* **1981**, 1372–1375.

(21) Doster, M. A.; Cotty, P. J.; Michailides, T. J. Evaluation of the atoxigenic *Aspergillus flavus* strain AF36 in pistachio orchards. *Plant Dis.* **2014**, *98*, 948-956.

(22) Abbas, H. K.; Zablotowicz, R. M.; Bruns, H. A.; Abel, C. A. Biocontrol of aflatoxin in corn by inoculation with non-aflatoxigenic *Aspergillus flavus* isolates. *Biocontrol Sci. Technol.* **2006**, *16*, 437-449.

(23) Abbas, H. K.; Wilkinson, J. R.; Zablotowicz, R. M.; Accinelli, C.; Abel, C. A.; Bruns, H. A.; Weaver, M. A. Ecology of *Aspergillus flavus*, regulation of aflatoxin production and management strategies to reduce aflatoxin contamination of corn. *Toxin Rev.* **2009,** *2-3,* 142-152.

(24) Accinelli, C.; Sacca, M. L.; Abbas, H. K.; Zablotowicz, R. M.; Wilkinson, J. R. Use of a granular bioplastic formulation for carrying conidia of a non-aflatoxigenic strain of *Aspergillus flavus*. *Bioresour. Technol*. **2009**, *100*, 3997-4004.

(25) Accinelli, C.; Abbas, H. K. New perspectives for the application of bioplastic materials in the biocontrol of *Aspergillus flavus* in corn. *Toxin Rev.* **2011**, *30*, 71-78.

(26) Accinelli, C.; Mariangela, M.; Sacca, L.; Vicari, A.; Abbas, H. K. Managing and monitoring of *Aspergillus flavus* in corn using bioplastic-based formulation. *Crop Prot.* **2012**, *32*, 30-35.

(27) Accinelli, C.; Abbas, H.K.; Vicari, A.; Shier, W. T. Aflatoxin contamination of corn under different agro-environmental conditions and biocontrol applications. *Crop Prot.* **2014**, *63*, 9-14.

(28) Accinelli, C.; Abbas, H. K.; Vicari, A.; Shier, W. T. Evaluation of recycled bioplastic pellets and a sprayable formulation for application of an *Aspergillus flavus* biocontrol strain. *Crop Prot*. **2015**, *72,* 9-15.

(29) Lin, M. T.; Dianese, J. C. A coconut-agar medium for rapid detection of aflatoxin production by *Aspergillus* spp. *Phytopathology* **1976**, *66*, 1466-1469.

(30) Saito, M.; Machida, S. A rapid identification method for aflatoxin-producing strains of *A. flavus* and *A. parasiticus* by ammonia vapor. *Mycoscience* **1999**, *40*, 205-211.

(31) Shier, W. T.; Lao, Y.; Steele, T. W. J; Abbas, H. K. Yellow pigments used in rapid identification of aflatoxin-producing *Aspergillus* strains are anthraquinones associated with the aflatoxin biosynthetic pathway. *Bioorg. Chem*. **2005**, *33*, 426-438.

(32) Accinelli, C.; Abbas, H. K.; Vicari, A.; Shier, W. T. Leaf application of a sprayable bioplastic-based formulation of a biocontrol *Aspergillus flavus* strain for reduction of aflatoxin contamination of corn. *Pest Manage. Sci.* **2016**, *72*, 1521-1528.

(33) Weaver, M. A.; Abbas, H. K.; Jin, X.; Elliott, R. B. 2016. Efficacy of water dispersible formulations of biocontrol strains of *Aspergillus flavus* for aflatoxin management in corn. *Food Addit. Contam.* **2016**, *F*, 346-351.

(34) The Use of Management Practices to Reduce Mycotoxin Contamination in Corn. https://content.ces.ncsu.edu/the-use-of-management-practices-to-reduce-mycotoxin-contamination-in-corn (Accessed: 13 April 2017).

(35) Aflaguard for Aflatoxin Prevention in Corn. http://news.utcrops.com/2012/05/aflaguard-for-aflatoxin-prevention-in-corn/ (Accessed: 13 April 2017).

525 **Figure 1.**
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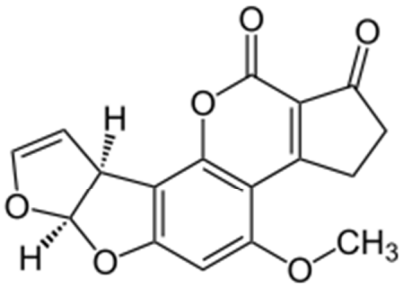

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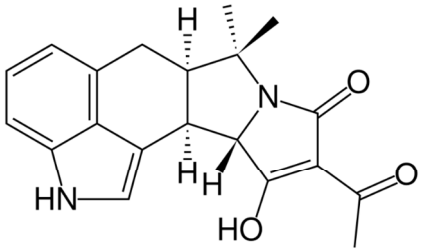

[2]

528 **FIGURE CAPTIONS:**
529
530 **Figure 1.** Chemical structures of the major aflatoxin component, aflatoxin B_1 **[1]** and
531 cyclopiazonic acid **[2]**.
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