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AGRICULTURAL AND FOOD CHEMISTRY



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

Hamed K. Abbas, Cesare Accinelli, and W. Thomas Shier

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1	Biological Control of Aflatoxin Contamination in US Crops and the Use of Bioplastic
2	Formulations of Aspergillus flavus Biocontrol Strains to Optimize Application Strategies
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24	ABSTRACT: Aflatoxin contamination has a major economic impact on crop production in
25	southern USA. Reduction of aflatoxin contamination in harvested crops has been achieved by
26	applying non-aflatoxigenic biocontrol Aspergillus flavus strains that can out-compete wild
27	aflatoxigenic A. flavus, reducing their numbers at the site of application. Currently, the standard
28	method for applying biocontrol A. flavus strains to soil is using a nutrient-supplying carrier (e.g.,
29	pearled barley for Afla-Guard). Granules of bioplastic (partially acetylated corn starch) have
30	been investigated as an alternative nutritive carrier for biocontrol agents. Bioplastic granules
31	have also been used to prepare a sprayable biocontrol formulation that gives effective reduction
32	of aflatoxin contamination in harvested corn kernels with application of much smaller amounts
33	to leaves later in the growing season. The ultimate goal of biocontrol research is to produce
34	biocontrol systems that can be applied to crops only when long-range weather forecasting
35	indicates they will be needed.
36	KEY WORDS: Aflatoxins, Aspergillus flavus, biocontrol, maize, peanuts, cottonseed, tree nuts.
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47 **INTRODUCTION**

Aflatoxin [1] contamination is a primary determinant of crop quality and hence value in corn 48 (maize, Zea mays L.) and other crops.¹ Aflatoxin contamination is a particular concern in the 49 50 southern tier of the US, affecting peanuts (groundnuts) in the east, corn (maize) across the south, 51 cottonseed in Arizona and Texas, and tree nuts in California. A major factor in the cost of 52 aflatoxin contamination to the producer are government regulations which limit crop usage based 53 on aflatoxin contamination levels. Specifically, the US Food and Drug Administration has 54 defined action levels for aflatoxin found in foods for direct human consumption, and feed for 55 dairy cattle and immature animals. Aflatoxin levels must be $\leq 20 \,\mu g/kg$ except in milk, for which 56 aflatoxin M1 must be $<0.5 \mu g/kg$; in feeds for breeding beef cattle, breeding swine, or mature 57 poultry aflatoxin levels must be $\leq 100 \ \mu g/kg$; in feeds for finishing swine aflatoxin levels must be $\leq 200 \ \mu g/kg$; in feeds for finishing beef cattle, aflatoxin levels must be $\leq 300 \ \mu g/kg$; and grain 58 59 with a flatoxin at $>300 \ \mu g/kg$ is suitable only for fermentation to produce ethanol. Additional 60 costs associated with aflatoxins in corn include the cost of assaying every commercial lot for 61 aflatoxins, and the cost of government-funded research conducted at universities and national 62 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.

69 In the US and other developed countries aflatoxin contamination of corn kernels almost 70 always occurs pre-harvest, because techniques have been implemented that effectively prevent 71 post-harvest production of aflatoxins, whereas techniques that effectively prevent pre-harvest 72 production of aflatoxins have proven to be much more difficult to develop. Typically corn 73 kernels are dried to less than 15% moisture content immediately after harvest, often with 74 propane-powered grain dryers, and then the kernels are placed under storage conditions that 75 continuously maintain a moisture level low enough to prevent A. flavus growth or aflatoxin 76 production. Government regulatory agencies assume that aflatoxin content in corn can be 77 assayed after harvest and the levels will not rise during storage. In developed countries this 78 assumption is generally valid, but in developing countries, where corn not meant for export is 79 often stored under conditions that are far from ideal, substantial post-harvest aflatoxin production 80 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.

88 Some success in reducing aflatoxin contamination has been obtained using agronomic 89 cultural methods.⁵ For many growing seasons, even in the southern US, aflatoxin contamination 90 of harvested corn is very low. However, when the weather is hot and dry during the kernel 91 filling period, particularly when night temperatures are greater than 68°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 fungus.⁸⁻¹³ Biocontrol fungal strains do not produce toxins, and must suppress multiplication of 101 102 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 in harvested corn kernels will occur when the biocontrol A. flavus strain takes over that 114

115 ecological niche and suppresses the number of wild, aflatoxigenic *A. flavus* conidia propagating116 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.

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124 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.

128 **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.⁸⁻¹⁰ 129 130 Aflatoxins are not a problem in cotton fiber production, but additional revenues from the sale of 131 cottonseed oil and cottonseed meal are a necessary part of the economics of cotton production. 132 Aflatoxins can be removed from cottonseed oil with a charcoal filter, but removal from 133 cottonseed meal is much more difficult. Cotty screened A. flavus isolates for inability to produce 134 aflatoxins, and he selected a non-aflatoxin producing strain (AF36) for study as a biocontrol 135 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 136 137 applied to the soil in the form of sterilized wheat kernels colonized by AF36. This application 138 technique resulted in suppression of aflatoxigenic A. flavus in treated soil and in domination of 139 the ecological niche by AF36. Substantial suppression of aflatoxigenic A. flavus by AF36 140 persisted in the soil into the following year. Reductions in aflatoxin contamination of the 141 cottonseed meal prepared from the crop ranged from 75-99%. Aspergillus flavus AF36, 142 manufactured by the Arizona Cotton Research and Protection Council was initially registered 143 with the Environmental Protection Agency (EPA) for cotton in Arizona in November, 2007. 144 Aspergillus flavus AF36 was also registered in February, 2012 with the EPA for use on cotton in 145 Arizona, and three counties in California and Texas.

146 Unfortunately, while AF36 did not produce aflatoxins, it did produce another mycotoxin, cyclopiazonic acid (CPA) [2] in peanuts¹¹ and in corn,¹⁴ which is a major concern for 147 commercial marketers of biocontrol A. flavus strains.¹⁵ Cyclopiazonic acid is a mycotoxin 148 149 structurally similar to ergot alkaloids that is produced by various *Penicillium* spp. and 150 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 151 152 pump in mammalian calciosomes, is considerably less toxic than aflatoxins but is still a concern 153 as a feed contaminant for young poultry, which are much more sensitive to it than mammals. Indeed, it has been suggested¹⁷ that the effects on turkeys that led to the discovery of aflatoxins 154 155 in the 1960s were actually caused by CPA produced with some aflatoxins by the A. flavus strain 156 that caused turkey "X" disease.

157 **Peanuts.** Peanut pods develop in the soil, where they are in direct contact with *A. flavus* and 158 other mycotoxin-producing fungi. The pods are particularly susceptible to infection by *A. flavus* 159 and *A. parasiticus* when subjected to drought during maturation of the kernels (i.e., late in the 160 growth season). Although irrigation during this period can prevent infection by *Aspergillus* spp.,

it is not available to most growers. Dorner¹² used mutants of A. parasiticus blocked in aflatoxin 161 162 biosynthesis to provide proof that non-aflatoxigenic isolates can displace A. flavus and A. 163 parasiticus in the soil, and reduce aflatoxin contamination of harvested peanut kernels. A wide 164 variety of A. flavus and A. parasiticus isolates were screened to identify isolates that did not 165 produce aflatoxins, CPA, O-methylsterigmatocystin, versicolorins or any other biosynthetic 166 intermediates. An isolate of A. flavus, NRRL 21882, designated Afla-Guard, emerged from a 167 comparison of isolates for their ability to reduce aflatoxins in harvested kernels and ultimately 168 was put forward for marketing as a commercial biocontrol strain. Extensive studies aimed at 169 developing a practical application process were conducted. Among the carriers investigated 170 were rice, pre-gelatinized corn flour granules and pasta bits. The final carrier selected was 171 pearled barley. All carriers tested were effective, but pearled barley had advantages in terms of 172 price and ease of manufacturing. Conidia suspensions were sprayed onto unsterilized pearled 173 barley. Large-scale field trials with Afla-Guard were conducted after its conditional registration 174 in 2004.

175 Corn. Biocontrol of aflatoxin levels in harvested corn kernels differs from biocontrol in 176 peanuts in several important ways. Most notably, corn kernels are located in an aerial part of the 177 plant, not in the soil. Furthermore, there are various infection mechanisms used by A. flavus to 178 colonize developing corn kernels, none of which are similar to the sites in peanuts. Silks appear 179 on developing corn ears in R1 about 65 days after planting of corn, and persist about 12 days, 180 when they darken and dry out. Wind-borne dust particles that carry A. flavus conidia can stick to 181 silks, where they germinate, then grow down the silk to the kernel to which the silk is attached, 182 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* 183

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.

186 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. 187 188 Studies began in the 2005 and 2006 growing seasons with the granular preparation containing 189 Afla-Guard applied to soil, and to whorls of the corn plant and as an aqueous suspension of 190 conidia, which was applied to silks of the corn plant four times during silking. In 2005, no 191 significant differences in aflatoxin contamination of harvested kernels were observed between 192 soil, whorl and silk application, but in 2006, whorl application was significantly better than soil 193 or silk application at reducing aflatoxin contamination in harvested kernels. This biocontrol 194 product, Afla-Guard, was registered with the Environmental Protection Agency (EPA) for corn, 195 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 196 197 in corn, in Texas and Arizona, is A. flavus AF36 manufactured by the Arizona Cotton Research 198 and Protection Council. It was initially registered with the EPA for cotton in Arizona in 199 November 2007 and the registration was expanded for the treatment of corn in Texas and 200 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 207 208 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 209 210 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 211 212 consecutive years (2008 to 2011). Reductions in aflatoxin contamination levels in harvested 213 pistachios of 20-45% were obtained. Aspergillus flavus strain AF36, manufactured by the 214 Arizona Cotton Research and Protection Council, was registered in February, 2012, with the US 215 Environmental Protection Agency (EPA) for use on pistachios in Arizona, California, Texas and 216 New Mexico.

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218 OPTIMIZATION OF AFLATOXIN BIOCONTROL IN CORN (MAIZE) IN 219 MISSISSIPPI AND ITALY

220 Initial studies on biological control of aflatoxins in corn in Mississippi used Afla-Guard, the A. 221 *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,} 222 ²³ This approach was successful enough to result in EPA registration as Afla-Guard for corn. 223 224 However, corn is different from peanuts in many ways, so that there was believed to be a 225 reasonable possibility that a corn-associated biocontrol A. flavus strain could be more effective 226 than a peanut-associated strain, the reason being that the infection site and the infection 227 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 228

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

231 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 232 233 physically breach the husk represents a significant alternate infection mechanism. Inoculating 234 soil with an A. flavus biocontrol strain is effective in reducing dust-borne wild-type aflatoxigenic 235 A. flavus spores reaching the silks to the extent that the biocontrol strain out-competes 236 indigenous A. flavus and replaces it in the soil reservoir. It is presumably the property of soil 237 competitiveness that makes Afla-Guard effective in controlling aflatoxin contamination in both 238 peanuts and corn. However, corn in principle offers opportunities to improve aflatoxin reduction 239 by applying the biocontrol A. flavus strain closer to the silks in distance and time, than is 240 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) nonaflatoxigenic *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 culture assays, the Lin and Dianese²⁹ test (vellow color on the back of colonies that produce 257 aflatoxins) and the Saito and Machida³⁰ test (red color produced on exposure to ammonia vapor) 258 259 were both empirical. They were therefore examined and shown to involve detection of the same pigments, which were biosynthetic precursors of aflatoxins.³¹ Confirmation of atoxigenicity was 260 261 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.

The resulting corn-associated non-aflatoxigenic biocontrol *A. flavus* strain NRRL 30797 (K49) was subjected to head-to-head comparisons with Afla-Guard and AF36 in a series of trials in corn in Mississippi between 2007 and 2009.²⁴ The results obtained indicated that the cornassociated non-aflatoxigenic strain K49 applied to the soil was not significantly better at reducing aflatoxins and cyclopiazonic acid in harvested corn kernels than Afla-Guard, but both K49 and Afla-Guard were significantly better than AF36.²⁴

Fungal Carrier. For soil application of biocontrol *A. flavus* strains to corn in Mississippi, initial studies used the standard pearled barley carrier used in studies on peanuts. Subsequent research has focused extensively on the use of starch-based bioplastic granules for soil application, field monitoring and as aqueous suspensions. Studies have been carried out with a 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 amylasecatalyzed 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 barley²⁴ 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.

288 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 289 290 soil remained near a typical 3.1 \log_{10} cfu/g in untreated plots, but plots treated with 15 and 30 291 kg/ha of biocontrol A. flavus on bioplastic granules experienced modest but significant increases 292 in total (i.e., aflatoxigenic plus non-aflatoxigenic) A. flavus over a 4-month period. However, the 293 percent aflatoxigenicity of isolates from treated plots fell steadily over a 4-month period from 294 about 40% to about 10%, whereas the percent aflatoxigenicity of isolates from untreated plots 295 did not change significantly. Soil application of biocontrol A. flavus at 15 kg/ha resulted in a 296 59% reduction in aflatoxin contamination in harvested corn kernels in 2009 and an 80% 297 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 aflatoxin produced by baited fungi and aflatoxin contamination of corn kernels was found.²⁵ 305

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 kg/ha.²⁶ The 2012 growing season was sufficiently hot and dry in both countries to provide a 308 309 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 $67 \pm 4.1\%$, whereas at 30 kg/ha it 313 reduced aflatoxins by an average of $94.8 \pm 5.3\%$. 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

321 APPLICATION SITE FOR BIOCONTROL A. FLAVUS

322 Almost all of the early research on biocontrol of aflatoxin contamination in various crops 323 have used a nutrient-rich carrier applied to the soil. In the case of peanuts, soil application is the 324 only reasonable option, because the harvested crop develops in soil, but for other crops that have 325 been subjects of aflatoxin biocontrol research, the harvested crop develops in aerial parts of the 326 plant – the ears in corn, the bolls in cotton and the seed inside a hard shell in tree nuts. In 327 principle, if a biocontrol agent is applied closer to or at its ultimate site of action, it should be 328 possible to apply it later and in smaller amounts. In the case of corn, the ultimate site of 329 interaction between the biocontrol A. flavus strain and aflatoxigenic soil-derived A. flavus is 330 believed to be the silks, but no one has developed a good way to apply biocontrol A. flavus to 331 corn silks. Applying biocontrol A. flavus to upper leaf surfaces of corn is closer to the ultimate 332 site of competition, and because total leaf surfaces are inherently smaller than the soil area, a 333 lesser number of biocontrol cfu should be needed. There are potential cost benefits from 334 applying smaller amounts, but the greatest potential benefits would come from reducing the 335 application lead time to less than, or equal to, the time of long-range weather forecasts.

Dorner¹³ compared application of biocontrol *A. flavus* strain Afla-Guard in pearled barley (a) 336 337 to soil at 22.4 kg/ha; (b) to plant whorls at 22.4 kg/ha and (c) as a conidial suspension with no 338 nutrient source sprayed from above, four times during silking in 2005 and 2006. In 2005 339 weather conditions resulted in low levels of aflatoxin contamination and no significant difference 340 from the control. However, in 2006 aflatoxin contamination of harvested corn was high in 341 untreated control corn and significantly reduced by all biocontrol treatments. Whorl application 342 gave the best results in the first 2006 planting, reducing contamination to about half the level of 343 that remaining after soil application of the same strain in pearled barley. The spraying conditions

344 used in the study were intermediate in effectiveness between whorl and soil application. In a 345 second planting, only whorl application significantly reduced aflatoxin contamination in 346 harvested corn kernels.

Accinelli et al.²⁸ developed a spravable formulation for biocontrol A. flavus strains using 347 348 finely divided pre-gelatinized corn starch-based bioplastic. Acetylation of starch to less than 349 10% acetate substantially alters the properties of the starch, reducing wettability to some extent, 350 but increasing adherence to cuticle-coated leaf surfaces and still allowing degradation by 351 amylases so that it can still provide nutrients to support growth of a biocontrol strain of A. flavus 352 or other biocontrol fungus. Another starch property that is retained is gelatinization by heating in 353 water at 80°C or higher. Gelatinization creates deformable particles that go through a sprayer 354 head better without sacrificing other desirable properties. These small particles can still act as a 355 nutrient source that allows a biocontrol fungus like A. flavus strain K49 to produce sufficient 356 conidia to compete with aflatoxigenic A. flavus from the soil reservoir. Although the small 357 particles support production of fewer biocontrol A. flavus conidia, the production is closer to the 358 site of competition (the silks) than is soil so it was expected to be sufficient.

359 In Northern Italy in 2012 weather conditions were hot and dry, favoring aflatoxin 360 contamination of harvested corn kernels. A 1% bioplastic-based formulation with A. flavus 361 strain K49 as the biocontrol strain, was sprayed on the leaves of corn growing on untreated soil 362 at one sixth the inoculum size normally used for soil application. Application of the sprayable 363 formulation resulted in an average 96.5% reduction in aflatoxin contamination of harvested corn 364 kernels relative to untreated control plots. An additional set of treatment groups had the soil 365 amended with untreated corn field plant material residues. This treatment resulted in slightly 366 higher aflatoxin contamination in kernels harvested from unsprayed controls, but leaf application

of the spravable biocontrol A. flavus formulation resulted in an average 97.1% reduction in 367 368 aflatoxin contamination of harvested corn kernels relative to unsprayed control plots.³² 369 Amending the soil with corn field plant material residues inoculated with aflatoxigenic A. flavus 370 NRRL 30796 further increased aflatoxin contamination in kernels harvested from unspraved controls. However, leaf application of the sprayable biocontrol A. flavus formulation resulted in 371 372 an average 96.9% reduction in aflatoxin contamination of harvested corn kernels relative to 373 unsprayed control plots. Examination of corn leaf surfaces after applying the sprayable 374 biocontrol A. flavus formulation indicated effective reduction in the percent aflatoxigenicity of 375 indigenous A. flavus relative to the unsprayed leaves of control corn plants. In contrast, spraying 376 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.³² 377

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.

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382 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 spravable bioplastic formulations of Afla-Guard and K49 are at 390 the planning stage.

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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 naturally occurring A. flavus on leaves, and the persistence of that dominance should be 403 determined. Studies on fungal DNA accumulated on leaves^{28,32} suggest that three weeks is 404 405 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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528 FIGURE CAPTIONS:

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530 **Figure 1.** Chemical structures of the major aflatoxin component, aflatoxin B₁ [1] and

531 cyclopiazonic acid [2].



