

Implications of Bt Traits on Mycotoxin Contamination in Maize: Overview and Recent Experimental Results in Southern United States

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ABSTRACT: Mycotoxin contamination levels in maize kernels are controlled by a complex set of factors including insect pressure, fungal inoculum potential, and environmental conditions that are difficult to predict. Methods are becoming available to control mycotoxin-producing fungi in preharvest crops, including Bt expression, biocontrol, and host plant resistance. Initial reports in the United States and other countries have associated Bt expression with reduced fumonisin, deoxynivalenol, and zearalenone contamination and, to a lesser extent, reduced aflatoxin contamination in harvested maize kernels. However, subsequent field results have been inconsistent, confirming that fumonisin contamination can be reduced by Bt expression, but the effect on aflatoxin is, at present, inconclusive. New maize hybrids have been introduced with increased spectra of insect control and higher levels of Bt expression that may provide important tools for mycotoxin reduction and increased yield due to reduced insect feeding, particularly if used together with biocontrol and host plant resistance.

KEYWORDS: *Bacillus thuringiensis*, aflatoxin, fumonisin, mycotoxin, Bt and non-Bt maize, environmental manipulation, transgenic corn, *Aspergillus flavus*, *Fusarium verticillioides*, insects, GM crops

■ INTRODUCTION

The Gram-positive spore-forming bacterium *Bacillus thuringiensis* was first isolated in 1902 in Japan as the causal agent of a silkworm disease¹ and later in Germany as a pathogen of flour moths. This bacterium was one of the first prokaryotic biological control agents to be used as a commercial insecticide in France in 1938. Since then, research in the area has led to more and widespread applications, and it has developed into an important agent for the control of insect pests worldwide. Since it was first introduced, other strains with variant forms of the endotoxin have been identified, which are effective against many insects. *B. thuringiensis* produces a protoxin, which accumulates an insoluble crystal protein of 130–140 kDa. This protoxin dissolves at the high pH values (up to 9.5) that are found in the midgut of some insects, and the soluble form of the protoxin is cleaved by proteases to form the active delta endotoxin of 60 kDa. The active toxin binds to the insect gut membranes, creating a pore resulting in loss of ions. Bt toxin is not toxic to humans or other animals because (i) the pH of vertebrate guts is too low to allow solubilization of toxin crystals and (ii) mammals, as well as nonsusceptible insect species, lack the brush border cell surface receptors for Bt found in susceptible insect species.² Thus, Bt toxin is safe for possible exposure to people in food or to animals in feeds.³ Over time, more than

200 types of Bt toxin produced by different strains of the bacterium have been identified, with these toxins having different activities and specificities across insect species.⁴

With advancements in genetic engineering,⁵ it eventually became feasible to move the gene that encodes the insecticidal toxins from *B. thuringiensis* into crop plants, including maize (*Zea mays* L., corn). When the gene is introduced, a tissue-specific promoter directs where the gene is to be expressed, thus producing toxin where plant damage typically occurs from targeted pests. Since the initial research breakthrough involving the transformation of tobacco plants to produce recombinants expressing Bt,⁶ subsequent work has developed the concept into an accepted method for protecting crops from insects, particularly lepidopteran pests. The first generation of Bt crops expressed the Cry1Ab gene, which confers resistance to some maize pests such as the European corn borer (*Ostrinia nubilalis* Hubner) with a single Bt gene. Subsequently, many other Cry

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proteins have been expressed in maize in an effort to broaden the range of economically important pests controlled. In addition to the Cry proteins, the vegetative insecticidal protein (Vip3A) is another protein secreted by *B. thuringiensis*, which has been expressed in maize to confer protection from lepidopteran pests. Like the Cry proteins, Vip3A is processed into its active form by cleavage in the insect gut, where it also forms ion pores. Vip proteins differ from the Cry proteins, sharing no sequence homology. They are expressed by *B. thuringiensis* during vegetative growth, rather than during sporulation like the Cry toxins. The assemblage of insects controlled by Vip toxin differs from those controlled by Cry toxins. The binding sites for the Vip proteins are distinct from those of the Cry proteins, thus making the evolution of insect cross-resistance less likely.^{7–9} Hybrids expressing Cry3 proteins control coleopteran pests.

■ IMPLICATIONS OF Bt TECHNOLOGY ON GLOBAL MAIZE PRODUCTION

Since its initial commercial introduction in the United States, APHIS and EPA have approved the use of 13 different Bt proteins in maize, which is more than for any other crop, for example, 5 for cotton and potatoes.^{10,11} Bt maize is grown in at least 18 countries worldwide (United States, Argentina, Brazil, Canada, Chile, Colombia, Honduras, Uruguay, Slovakia, Spain, Mexico, Poland, Portugal, Czech Republic, Romania, South Africa, Egypt, and The Philippines).¹² In Europe, Spain was the first European country to utilize Bt maize in 1997 and has the greatest acceptance of Bt maize by farmers.¹³ As of 2009, Spain's production exceeded 76000 ha, whereas the Czech Republic, Portugal, Romania, and Spain cultivated <7000 ha of Bt maize.¹⁴ Bt maize was briefly grown in France and Germany, but these countries subsequently suspended its use. Most production in the Eastern European countries is not irrigated and is less likely than maize cultivated in the southern United States to be exposed to the extremely warm temperatures that favor aflatoxin contamination. Thus, mycotoxin contamination is relatively unlikely in Eastern Europe. The area with the greatest potential for Bt maize to reduce mycotoxin contamination is Africa. Some African countries have begun preparing for commercial introduction of bioengineered crops.^{15–17} Studies in South Africa¹⁸ and Kenya¹⁹ indicate a potential for farmers' acceptance, and the potential for aflatoxin and fumonisin contamination in sub-Saharan Africa is high.²⁰ Bt maize in Kenya controlled the population of destructive stem borers.²¹ In China, the use of Bt cotton caused a decrease in the use of insecticides by up to 80%.²² Indian farmers have also been helped by the decreased need for pesticides in their small cotton fields.²³ There has been a backlash against genetically modified crops such as Bt maize. More advanced countries such as the European Union, Japan, and Australia have issues with GM crops, which are related to concerns about the environment.²⁴

■ MAJOR MYCOTOXINS AFFECTING MAIZE

Aflatoxins are the most toxic secondary metabolites commonly found in maize. Aflatoxins are produced by *Aspergillus* spp., predominantly *Aspergillus flavus*. The amount of aflatoxins in crops is regulated by governments, including that of the United States, which sets the maximum aflatoxin level for direct human consumption at 20 ppb. Aflatoxin is known to cause liver cancer in humans and acute toxicity and death in poultry.^{25,26} Cyclopiazonic acid (CPA) is also produced by *A. flavus*, and it is

often found along with aflatoxins in animal feeds. CPA causes a disturbance of calcium metabolism in muscles, often causing death in animals that ingest sufficient quantities. CPA is also produced by *Penicillium cyclopium*.^{27–29}

Fumonisin are produced by *Fusarium* spp. such as *Fusarium verticillioides* and *Fusarium proliferatum*. They are known to cause leukoencephalomalacia in horses and other equine species and pulmonary edema in swine. The FDA has set advisory or guidance levels for fumonisins in maize and maize products at 2–4 ppm.^{26,30} Maize plants stressed by heat and drought are susceptible to simultaneous infection by *A. flavus* and *Fusarium* spp., resulting in kernels contaminated with both aflatoxins and fumonisins. *Fusarium* spp. also produce other mycotoxins including zearalenone, which causes vulvaginitis (hyperestrogenism) in swine, and deoxynivalenol (DON), which causes feed refusal, nausea, and vomiting.²⁶

Insect infestation may promote fungal inoculation and subsequent mycotoxin contamination in several ways. Insects burrowing through the husks or down the silk channel can open infection routes for air- or dustborne fungal pathogens. Fungal propagules may also be carried to developing kernels directly on the bodies of insect vectors. Mycotoxin levels may also be affected indirectly through a reduction in plant health resulting from insect damage.

■ INSECTS' ROLE IN MYCOTOXIN CONTAMINATION

Many of the insects that are managed by Bt toxins may act as vectors for fungal pathogens of maize and other plants.³¹ Some of these fungi produce economically important mycotoxins (Figure 1), notably aflatoxins and CPA by *Aspergillus* spp., and fumonisins, zearalenone, and DON by *Fusarium* spp. It has always been expected that Bt maize would be less susceptible to mycotoxin-producing plant pathogens because of vector control. Fungal pathogens may be introduced to a developing ear by insect feeding that occurs before the Bt toxin affects the insect. A model has been developed to assess the economic and health benefits of adopting Bt maize on reducing aflatoxin and fumonisin contamination,³² including a cost benefit of \$23 million annually in the United States, with further benefits in China and Argentina.

The most important insect pests in a particular region change from year to year, but the following are some of the insects most commonly associated with mycotoxin contamination of maize. European corn borer (*O. nubilalis*) (ECB) is one of most economically important insect pests in the major maize-producing areas of the United States. The greatest damage comes from feeding in the whorl and burrowing into the stalk or ear shank. In the southern United States, larvae enter diapause and overwinter primarily in the stalks. Adults emerge as moths in the spring and may travel great distances before mating and laying eggs on the upper surface of maize leaves. Depending on the climate and the ECB ecotype, one, two, three, or more generations may occur in a season. The later generations are ear feeders and are often found in diseased maize ears.³³ Transgenic maize may be protected against ECB, although not all types of Bt toxins confer the same level of protection.³⁴

The life cycle of the southwestern corn borer (SWCB) is similar to that of the ECB. The relative importance of each insect species depends on many factors, but the role of SWCB in increasing aflatoxin contamination was clearly proven by Windham et al.³⁵ In that study, the presence of SWCB undermined the genetic resistance to aflatoxin contamination of the experimental breeding lines. The sugar cane borer, *Diatraea*

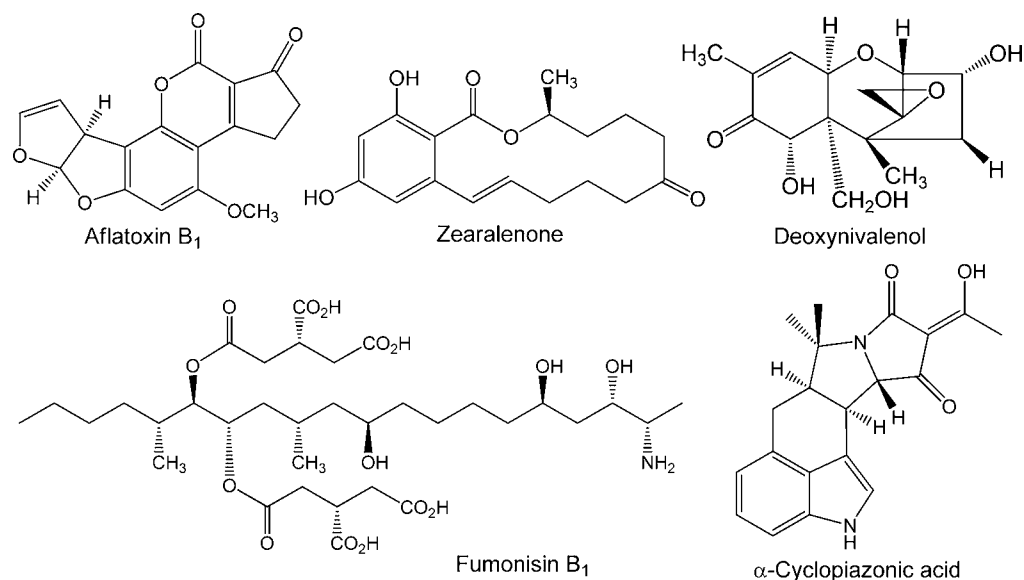


Figure 1. Chemical structures of mycotoxins mentioned in the text.

saccharalis, feeds on maize in a similar way to ECB and SWCB and is the most common borer pest of maize in limited areas of the Mid-South United States, for example, areas of Louisiana and Texas.^{36–38} Bt-maize is the most effective method for control of this pest.³⁶ The western bean cutworm (WBC) feeds on maize reproductive tissues and leaves and is thought to introduce *Aspergillus* spp. into the maize ear during feeding. Whereas the Cry1F and Vip3Aa20 proteins protect against WBC, the other Bt toxins offer limited or no protection.^{34,39,40}

Corn earworm (*Helicoverpa zea*) (CEW) is an insect pest of secondary importance in most years. Many other crops and weedy plants are also hosts for CEW. Larvae of CEW overwinter in the soil, mostly in the southern United States. Emerging moths are highly mobile, traveling with storm fronts over great distances before mating and leaving eggs on maize whorls and silks. After larval development in the ear, they drop to the ground and pupate. Depending on environmental conditions, there can be many generations in one season.⁴¹ Several “first-generation” Bt maize varieties provided some CEW suppression, but varieties containing the events MON89034 or MIR 162 are highly protected from CEW infestation.

The corn rootworm complex (*Diabrotica* spp.; Coleoptera: Chrysomelidae) causes \$1 billion annual losses to maize in the United States,⁴² with this damage due to larval feeding on roots. The western corn rootworm, *Diabrotica virgifera* LeConte, and northern corn rootworm, *Diabrotica barberi* Smith and Lawrence, cause the most damage to maize roots in the U.S. Corn Belt. Severe silk clipping by adult corn rootworm beetles may reduce yield because of poor pollination, but such reductions occur only if beetle densities are unusually high at silking and environmental conditions are negatively affecting the rate of silk growth. For example, up to 20 adult beetles per maize ear did not affect irrigated maize yields in Colorado,⁴³ whereas Kuhlman⁴⁴ attributed yield losses to as few as 5 beetles per plant in nonirrigated maize. Studies associating mycotoxin contamination of maize with rootworm beetle damage are uncommon. However, Gilbertson et al.⁴⁵ did observe higher levels of stalk rot fungal infection in maize infested with western corn rootworm beetles, compared to maize not heavily infested, and demonstrated that the beetle vectored *Fusarium moniliforme* and *Fusarium subglutinans*. Root feeding by western

corn rootworm (WCR) larvae resulted in a 50-fold increase in colonization of maize root by *Fusarium verticillioides*.⁴⁶ This could enhance the occurrence or level of fumonisin in maize by this endophytic pathogen. An effective Bt transgene would need to be expressed in root tissues early in plant development to prevent these infections vectored by WCR.

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith), is another economically important pest in maize, although it is not considered a primary target for insect resistance management by the U.S. Environmental Protection Agency. Hardke et al.⁴⁷ showed that Bt-maize hybrids expressing Cry1Ab or Cry1F significantly reduced growth, development, and survivorship of fall armyworm compared to those offered non-Bt maize tissue.

■ ECOLOGY AND ETIOLOGY OF MYCOTOXIN-PRODUCING FUNGI

A summary of the ecology of *A. flavus* in maize fields is illustrated in Figure 2. *A. flavus* is ubiquitous and survives as a saprophyte in soil and plant residues residing as mycelia, conidia, and sclerotia (see Figure 2).³¹ Maize residues left on the soil surface under no-tillage conditions, particularly unharvested kernels, can support the growth of mycotoxin-producing fungi during fall and spring, and this growth represents a major source of inoculum.⁴⁸ Sclerotia allow fungi to survive under harsh environmental conditions,^{49,50} including survival over the winter. Thus, it is the sclerotia that have colonized plant residues in the fall, which successfully overwinter and are available to dominate the soil reservoir in the spring. As a result, they may be important sources of contamination in the form of dustborne conidia being deposited on emerging crops and weeds.⁴⁸

F. verticillioides is widely found as a seedborne contaminant in maize, from which it readily colonizes seedlings, and it persists in maize from generation to generation as an endophyte. *F. verticillioides* can survive in crop residues and in soil, and its spores are commonly airborne on dust in maize production areas,⁵¹ but dustborne spores have been reported to represent a minor infection route for maize kernel.⁵² *Fusarium* ear rot typically results from insect feeding, similar to the case with *A. flavus*. In particular, thrips may enter the silk channel after pollination, carrying *Fusarium* spores that infect the ear,⁵³ and

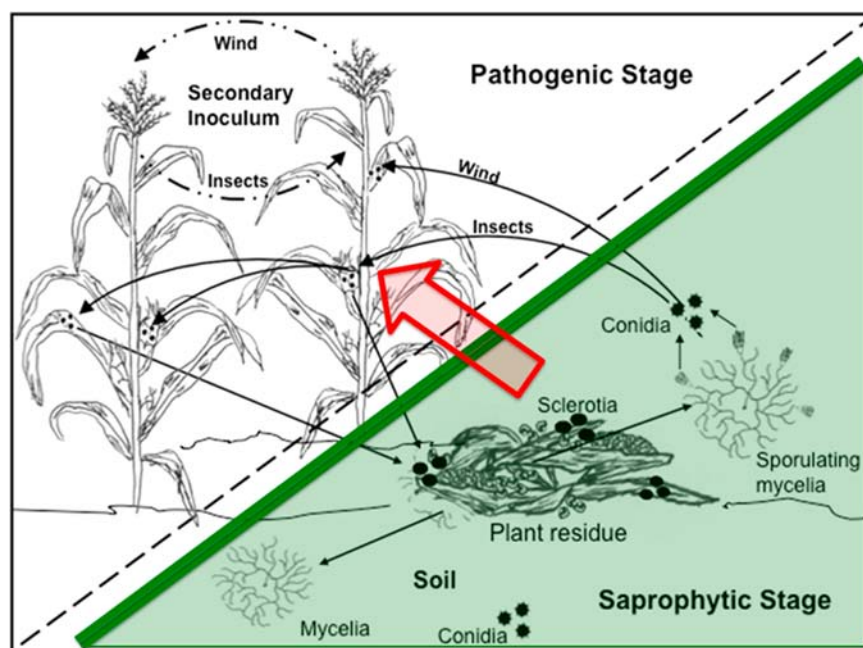


Figure 2. Schematic representation of key elements in *Aspergillus flavus* ecology. Adapted from Abbas et al.¹¹

can be a factor in fumonisin contamination.⁵⁴ However, the use of Bt transgenes would not be effective in controlling fumonisin contamination vectored by thrips.

Munkwold et al.⁵⁵ showed that *F. verticillioides* inoculated on silk was found in 75–90% of isolates in grain, versus 0–30% when the fungus was inoculated on seed prior to planting, indicating the importance of dustborne contamination during the silking phase of maize growth. In southern zones, early planting can aid in reducing *Fusarium* ear rot by allowing silking to occur before the start of hot, dry conditions that stress plants and favor fungal growth. As the growing season progresses, the intensity of insect populations also increases; high densities of fall armyworm is a limitation to late-planted maize in southern areas.

Insects are major vectors for *Aspergillus* spp. and *Fusarium* spp. entry into maize ears. For example, documentation exists associating ear-feeding insect damage with increased aflatoxin levels in preharvest maize.^{40,56–58} Therefore, reducing insect damage by planting crops expressing the appropriate Bt gene may reduce mycotoxin contamination. Because mycotoxin contamination in maize is heavily influenced by the environment, associating insect damage to maize ears in the field with subsequent mycotoxin contamination can be difficult. As discussed in more detail below, several authors have successfully shown that Bt maize hybrids successfully reduced aflatoxin and fumonisin contamination of the grain.^{59–61}

■ MAIZE Bt HYBRIDS

In the years since the introduction of Bt transgenic, the biotechnology industry has continued to produce new Bt varieties by introducing new insecticidal proteins and moving these genes into elite maize genotypes.^{10,11} Table 1 summarizes the toxins presently available and highlights the varying spectrum of insects controlled by each package of Bt toxins. Transgenic herbicide resistance is inherently linked with Bt insect resistance in genetically modified plants, as the transgenes for both traits are commonly moved in a single genetic engineering event. Whereas individual Cry or Vip toxins may provide a

limited spectrum of insect control, genes for more than one toxin may be “stacked” in transgenic maize to widen the spectrum of insect resistance. Up to six insecticidal proteins have been included in hybrids presently available. To forestall the emergence of insect resistance to any or all of the Bt traits, the biotechnology industry has developed three antiresistance strategies. First, the industry has advocated the introduction of Bt maize in the context of a sound overall insect management program, including crop rotation and appropriate use of seed, soil, and foliar insecticides. Second, there is a regulatory requirement to allocate up to 20% of land area, and 50% in cotton-growing regions of the United States, depending on the particular Bt trait, to non-Bt maize, allowing insects a refuge free from Bt selection pressure.⁶² The ideal size, shape, and placement of a refuge are reviewed elsewhere.^{63,64} An interesting recent development is the regulatory acceptance of “refuge in the bag,”^{65,66} in which non-Bt seed can be blended and interplanted with the Bt hybrid, thus ensuring the timely planting of the refuge and greatly adding to the convenience for the farmer. Finally, multiple insect toxins that control the same insect target can be “pyramided” in a single hybrid, negating the selective advantage of resistance to any one particular Bt toxin.

The high adoption rate of Bt technology by U.S. maize farmers, coupled with its high efficacy, has resulted in an area-wide suppression of the dominant North American maize insect pest, the European corn borer (ECB). Prior to Bt maize, timing of sprays was difficult in the management of ECB. The economic benefit of this control in the upper Midwest was estimated to be \$6.9 billion between 1996 and 2009, including \$4.3 for non-Bt maize acres, which benefited from the Bt-derived ECB population suppression.⁶⁷

■ Bt MAIZE AND FOOD SAFETY ISSUES WITH GENETICALLY MODIFIED PLANTS

Bt maize was originally approved as being safe for human/animal consumption on the basis of rat feeding studies conducted by Monsanto and reviewed by the European Food Safety Authority (EFSA) Genetically-Modified Organisms Panel Working Group.⁶⁸

Table 1. Commercially Available Transgenic Maize Traits^a

trade name ^c	transformation event	foreign genes expressed ^d	herbicide tolerance ^e	insects controlled ^b									
				ECB	CRW	CEW	FAW	WBC	BCW	SB			
Agrisure GT	GA21	Zm EPSPS	glyphosate										
RoundUp Ready 2	NK603	CP4 EPSPS	glyphosate										
LibertyLink	T25	PAT	glyphosate										
Agrisure CB, Yieldgard CB	MON810	Cry1Ab	glufosinate	X									
Agrisure RW	MIR 604	Cry3A	glufosinate		X								
Herculex	TC1507	(PAT, Cry1F)	glufosinate	X			X		X				
Herculex RW	DAS-59122-7	(PAT, Cry3A, Cry35Ab1)	glufosinate	X				X					X
Herculex XTRA	TC1507 + DAS-59122-7	(PAT, Cry1F) + (PAT, Cry3A, Cry35Ab1)	glufosinate	X				X					X
Agrisure CB/LL/RW	Bt11 + MIR604	(PAT, Cry1Ab) + Cry3A	glufosinate	X									
Agrisure GT/RW	GA21 + MIR 604	Zm EPSPS + Cry3A	glyphosate	X									
Agrisure CB/LL	Bt11	(PAT, Cry1Ab)	glyphosate, glufosinate	X									
Agrisure GT/CB/LL	GA21 + Bt11	Zm EPSPS + (PAT, Cry1Ab)	glyphosate, glufosinate	X									
Agrisure 3000GT	GA21 + Bt11 + MIR 604	Zm EPSPS + (PAT, Cry1Ab) + Cry3A	glyphosate, glufosinate	X						X			
Genuity VT Double Pro	NK603+ MON89034	CP4 EPSPS + (Cry1A.105, Cry2Ab2)	glyphosate	X				X					
Genuity VT Triple	MON88017 + MON810	(CP4 EPSPS, Cry3Bb1) + Cry1Ab	glyphosate	X						X			
Genuity VT Triple Pro	MON88017 + MON89034	(CP4 EPSPS, Cry3Bb1) + (Cry1A.105, Cry2Ab2)	glyphosate	X				X		X			
SmartStax	MON88017 + MON89034 + DAS-59122-7 + TC1507	(CP4 EPSPS, Cry3Bb1) + (Cry1A.105, Cry2Ab2) + (PAT, Cry3A, Cry35Ab1) + (PAT, Cry1F)	glyphosate, glufosinate	X				X		X			X
Agrisure Viptera 3110	Bt11 + GA21 + MIR162	(PAT, Cry1Ab) + Zm EPSPS+ Vip3Aa20	glyphosate, glufosinate	X				X		X			X
Agrisure Viptera 3111	Bt11 + GA21 + MIR162 + Cry3A	(PAT, Cry1Ab) + Zm EPSPS + Vip3Aa20 + Cry3A	glyphosate, glufosinate	X				X		X			X
Agrisure Viptera 3122	Bt11 + GA21 + MIR604 + DAS-59122-7	(PAT, Cry1Ab) + Zm EPSPS + Cry3A + (PAT, Cry3A, Cry35Ab1)	glyphosate, glufosinate	X				X		X			X
Agrisure Viptera 3220	Bt11 + GA21 + MIR162 + TC1507	(PAT, Cry1Ab) + Zm EPSPS + Vip3Aa20 + (PAT, Cry1F)	glyphosate, glufosinate	X				X		X			X

^aTable adapted from Buntin,²⁰ Proost and Bollman,²⁵ and DiFonzo and Cullen.⁴⁷ ^bECB, CRW, FAW, WBC, BCW, and SB indicate control of European corn borer, corn rootworm, fall armyworm, western bean cutworm, black cutworm, and stalk borer, respectively. Insects not listed may be suppressed or partially controlled. ^cRoundUp Ready 2, Yieldgard, Yieldgard VT Triple, Yieldgard VT Triple Pro, and Genuity SmartStax are trademarks of Monsanto. Herculex is a trademark of Dow Agroscience. Agrisure and Viptera are trademarks of Syngenta. ^dGenes listed within parentheses are a single transformation event. ^eGlyphosate tolerance allows in-season application of herbicides such as RoundUp, Touchdown, and Accord. Glufosinate tolerance allows in-season application of herbicides such as Liberty.

Subsequently, a range of government, academic, and industrial scientists have affirmed the long-term safety of genetically modified plants,^{69,70} including a three-generation study of the feeding of Bt maize to rats by Kilic and Akay.⁷¹ Government regulatory agencies continue to carefully scrutinize existing and new transgenic plant introductions to ensure public safety. In fact, Bt maize is being grown worldwide on a vast scale with clear, observable, measurable positive effects on the environment and human health through reduced insecticide use, better and more selective pest control, higher yields, and improved yield stability.^{67,72,73}

■ ECOLOGICAL IMPACT OF Bt MAIZE

Two ecological concerns with Bt maize that have attracted attention are the possibility of nontarget toxicity, particularly to monarch butterflies, and the eventual development of resistance to Bt maize by target insect species.⁷⁴ Other secondary concerns include the following: (i) reduction in crop plant genetic diversity; (ii) potential horizontal transfer and recombination of Bt genes into weeds to create “superweeds” resistant to their natural enemies; (iii) potential vector-mediated horizontal transfer and recombination of Bt genes to create new strains of bacteria pathogenic to maize and other plants; and (iv) consideration for unnecessary additional cost of production.

Concern about the effect of Bt maize fields on monarch butterflies began with the publication of a report by Losey et al.⁷⁵ that the pollen of Bt maize fed on milkweed leaves was toxic to monarch butterfly larvae in the laboratory. Dust containment of the Bt transgene was an issue of regulatory concern. A more definitive field study by Sears et al.⁷⁶ was carried out in several U.S. states and Canada and found that the impact of Bt maize adoption at the levels then had a negligible impact on monarch butterfly populations. One reason given was that commercial varieties of Bt maize express very little Bt in the pollen.

Concern among the various aspects of the agricultural industry about the development of resistance to Bt maize by target insect species⁷⁷ has always been considered inevitable by most of the various stakeholders in American agriculture.⁷⁴ Carrière et al.⁷⁸ described the development of field-evolved resistance to Bt crops in only five Lepidoptera pests during the past 14 years, and Gassmann et al.⁷⁹ reported the development of field-evolved resistance of a Coleoptera (western corn rootworm), but in general there have been surprisingly few reports of field-evolved resistance to Bt crops. It is not clear whether this is the result of inherent barriers to developing resistance to crop-expressed Bt or the early adoption and success of refuge strategies⁸⁰ for the prevention of resistance.

■ REVIEW OF FIELD TRIALS ASSESSING THE IMPACT OF THE GENETICALLY MODIFIED Bt EVENT ON MYCOTOXIN CONTAMINATION IN MAIZE

Aflatoxins. Given the long-standing recognition of the relationship between insect damage and mycotoxin contamination and insect control provided by Bt maize, published data to support the hypothesis that Bt maize yields harvested kernels with lower aflatoxin levels is surprisingly equivocal. Williams et al.⁸¹ found reduced aflatoxin levels when experimental test-crosses included Bt lines. Wiatrak et al.⁸² observed decreased aflatoxin in Bt hybrids in one year, but not in two subsequent years. While studying the effect of Bt hybrids on WBC and ECB infestation, Catangui and Berg⁴⁰ found no significant

effect of Bt on aflatoxin and variable effect of Bt on fumonisin content. Abbas et al.³¹ failed to find an effect of Bt on aflatoxin levels in observations of commercial fields over 3 years including 65 hybrids. Numerous field trials with Bt hybrids have also failed to demonstrate a significant field-level reduction in aflatoxin concentration (Bibb et al., 2012, personal communication).⁸³

One plausible explanation for a lack of protection against aflatoxin contamination by Bt maize might be that the “Bt” hybrids evaluated may have offered little or no protection against the insects that are important for spreading *Aspergillus* infection in that particular field (e.g., corn earworm or fall armyworm). Another plausible explanation is that, whereas some of the more recently developed Bt hybrids have exceptional resistance to many insects, the insects may still live long enough after consuming Bt maize tissue to vector *A. flavus* to additional plants or to create routes in them for dustborne *A. flavus* to infect ears. Another explanation is that insects do not play as large a role in vectoring *Aspergillus* as is widely believed and that other infection mechanisms, such as dustborne *Aspergillus* landing on silks, usually outweigh insect-vectoring effects on aflatoxin contamination in harvested kernels.

Fumonisin. The evidence for mycotoxin reduction in harvested kernels of Bt maize is clearer for *Fusarium* mycotoxins. Even the first-generation Bt maize hybrids are known to resist *Fusarium* ear rot and are associated with lower levels of fumonisin contamination in harvested kernels.^{60,84} A recent meta-analysis concluded that Bt maize exhibited reduction in one or more *Fusarium* mycotoxins in 19 of 23 field studies.⁸⁵ There is a reporting bias against finding “no effect”, and contrary evidence is often attributed to extreme weather events or other external factors.⁸⁶ Nevertheless, the preponderance of evidence appears to be in support of the hypothesis that the widespread adoption of Bt technology is reducing the occurrence of fumonisin in harvested maize kernels.

The initial report of maize expressing the Bt trait (*cry1Ab*) reducing fumonisin contamination was that of Munkvold et al.⁸⁴ This study examined Bt hybrids expressing the *cry1Ab* gene in various tissues when noninoculated or artificially infected with the European corn borer (ECB; *O. nubilalis* Hubner). Maize expressing the Bt toxin in kernels was effective in reducing ear rot by 58–87% and kernel infection by 17–39% in three years of study. Maize expressing the *cry1Ab* gene in other tissues was not effective in reducing *Fusarium* infection. Subsequent studies⁶⁰ confirmed that hybrids expressing *cry1Ab* or *cry9c* in kernels reduced fumonisin contamination of harvested kernels from 16.5 mg kg⁻¹ in control hybrids to 2.1 mg kg⁻¹ in the Bt hybrids.

Field studies to control fumonisin by Bt maize have been conducted in The Philippines and in Argentina by De La Campa et al.,⁸⁷ who evaluated several Bt maize hybrids (*cry1Ab* gene), comparing them to their non-Bt conventional isolines. In the Argentinian trials, three pairs of Bt hybrids and their respective isolines were evaluated in four locations in 2001 and 2002, whereas the Philippine trials evaluated two pairs of Bt hybrids with their respective isolines in two locations. Insect damage was calculated on the basis of the average number of holes per plant in Argentina, whereas in The Philippines it was estimated by the length of the hole at harvest. In all trials a significantly lower level of insect damage ($P < 0.001$) was reported for the Bt hybrids compared to their isoline counterparts (Table 2). A model was constructed to explain the variance in fumonisin accumulation in harvested kernels. Location alone accounted for almost half the variation (47%).

Table 2. Influence of Bt Trait (Cry1AB) Compared to Its Conventional Non-Bt Isoline on Fumonisin Levels and Insect Damage of Maize in Argentinian and Philippine Field Trials Conducted in 2000–2002^a

location	year	hybrid	fumonisin (mg kg ⁻¹)	insect damage
Argentina	2000	Bt	2.46 ^b	0.01 ^b
		non-Bt	6.29	2.17
	2001	Bt	0.56 ^b	0.01
		non-Bt	3.06	1.20
Philippines	2001	Bt	0.81	0.00 ^b
		non-Bt	0.97	0.78
	2002	Bt	0.25	0.00 ^b
		non-Bt	0.45	1.39

^aTable adapted from De La Campa et al.⁷² ^bMeans of fumonisin levels and insect damage in Bt hybrid significantly lower than conventional isoline.

A total of 82% of the variance was explained by a complex model, which incorporated insect damage level and multiple rain and temperature parameters. A significant reduction in fumonisin levels attributed to the Bt transformation was observed in Argentina, but not in The Philippines. The drier environmental conditions in Argentina compared to The Philippines favored *Fusarium* spp. infection and fumonisin accumulation.

Influence of the Bt trait on fumonisin incidence was measured in nationwide field trials by Hammond et al.⁸⁸ In this study they examined a total of 210 field trials conducted at 107 locations at U.S. universities between 2000 and 2002. In the 210 trials conducted, 126 had fumonisin levels >2 mg kg⁻¹ (the FDA advisory level) in non-Bt maize. Of the 126 trials, the fumonisin levels from Bt maize were under the action limit for 58. These field trials conducted in a wide range of environmental conditions suggest that maize expressing the (*cry1Ab* gene, Monsanto transformation) trait can effectively reduce fumonisin contamination, especially when the dominant insects are corn borers (*Papilio nebris* Quenee; *Diatraea grandiosella* Dyer; and *O. nubilalis*). However, the *cry1Ab* gene is only moderately effective when the insect pressure is the corn earworm. Aflatoxin was detected in only limited trials, whereas DON was found at similar levels in Bt maize and control hybrids; no zearalenone was detected.

Fusarium Mycotoxins. Strip field trials conducted for four years in Ontario, Canada, assessed the effect of the Bt trait in maize on fumonisin and DON levels.⁸⁹ Average fumonisin levels were <0.25 mg kg⁻¹ with no effect of the Bt trait. However, significant reductions in DON in maize expressing the Bt trait were observed compared to the non-Bt isoline in three of four years (Table 3). The average reduction in DON over the four years was 38%, in which 65% of grain from Bt maize was positive for DON compared to 84% positive in the non-Bt isoline. The accumulation of DON among both maize hybrids was highly correlated with a high incidence of ECB damage.

Extensive field trials conducted in 21 sites in southern France⁹⁰ assessed the effect of the Bt (*cry1Ab* gene from the MON 810 event) trait compared to its non-Bt isoline in a relatively homogeneous environment in 2005 and 2006. Grain was analyzed for fumonisin, DON, and zearalenone by LC-MS-MS. As summarized in Table 4, fumonisin was significantly ($P < 0.0001$) reduced by 92–96% in the Bt hybrid compared to

Table 3. Influence of a Bt Gene Compared to Conventional Isoline (Non-Bt) on Deoxynivalenol Average Concentrations in Ontario Strip Trials^a

year	no. of trials	fumonisin level ^b (mg kg ⁻¹)		<i>P</i> ^c
		Bt	non-Bt	
1996	17	0.45	1.25	0.0001
1997	27	0.36	0.51	0.02
1998	31	0.69	1.15	0.0019
1999	27	1.06	1.19	0.51

^aTable adapted from Schaafsma et al.⁷⁴ ^bConcentrations are expressed as detransformed means from $\ln(\text{Don} + 0.10)$. ^cA highly significant year \times Bt interaction was observed.

Table 4. Occurrence of Fumonisin, Deoxynivalenol (DON), and Zearalenone in Maize Expressing the *cry1Ab* Bt Gene and Its Non-Bt Isoline in Southern France Field Trials^a

mycotoxin	year	Bt	non-Bt	<i>P</i>
fumonisin (mg kg ⁻¹)	2005	0.26 \pm 0.114	6.12 \pm 1.3	<0.0001
	2006	0.43 \pm 0.25	5.62 \pm 1.5	<0.0001
DON (mg kg ⁻¹)	2005	0.19 \pm 0.05	0.11 \pm 0.06	0.040
	2006	0.98 \pm 0.47	0.24 \pm 0.06	0.010
zearalenone (μ g kg ⁻¹)	2005	9.37 \pm 3.03	18.95 \pm 8.86	0.240
		1.57 \pm 1.42	3.47 \pm 2.31	0.270

^aTable adapted from Folcher et al.⁷⁵ Results are the mean of five replicates \pm standard deviation.

its non-Bt isoline. However, in both years DON was significantly greater ($P = 0.040$ in 2005 and $P = 0.010$ in 2006) in the Bt maize compared to its non-GM isoline (63.2% in 2005 and 308% in 2006). Although the average zearalenone level in the Bt hybrid was \sim 50% lower, the difference was not significant. The inverse effect on fumonisin and DON is consistent with a competitive interaction among various *Fusarium* spp., particularly *F. verticillioides* producing fumonisin and *F. graminearum* producing DON.

■ USDA-ARS FIELD TRIALS IN THE U.S. MID-SOUTH

The role of Bt-based insect control on mycotoxin contamination in harvested maize kernels has been evaluated at Marianna, Arkansas, over three growing seasons.⁹¹ In 1998 levels of natural mycotoxin contamination of harvested kernels were evaluated in 17 conventional maize hybrids and 4 Bt hybrids. In 1999, 17 conventional and 12 Bt hybrids were evaluated, and in 2000 7 conventional hybrids and 2 Bt hybrids were evaluated. In 1998, the levels of insect damage and *Fusarium* ear rot were significantly lower ($P < 0.01$) in Bt compared to non-Bt hybrids (\sim 50% lower), whereas levels of *Aspergillus* ear rot were similar (Table 5). In all three years no significant difference in aflatoxin levels was observed between harvested kernels of Bt and non-Bt hybrids. In 1998, harvested kernels from four non-Bt and nine Bt hybrids had aflatoxin levels below limits of detection. Fumonisin contamination of harvested kernels was significantly lower in Bt hybrids compared to conventional hybrids only in 1998. In 1998 levels of fumonisin were <10% of that observed in 1999 and 2000, suggesting that the Bt gene in maize may be effective only under conditions of low *Fusarium* infection potential.

The effect of Bt-expressing maize hybrids on aflatoxin and fumonisin levels in harvested kernels compared to nontransformed

Table 5. Levels of Aflatoxin and Fumonisin in Non-isogenic Maize Grain Observed in Marianna, Arkansas, Field Trials Comparing Bt and Non-Bt Hybrids in 1998, 1999, and 2000^a

year	hybrid	no. of hybrids	aflatoxin levels total ($\mu\text{g kg}^{-1}$)	fumonisin ^b (mg kg^{-1})
1998	non-Bt	16	227 \pm 5	45.6 \pm 6.0
	Bt	5	196 \pm 9	39.6 \pm 4.2
1999	non-Bt	15	27 \pm 8	3.32 \pm 0.60 ^b
	Bt	14	4 \pm 2	2.09 \pm 0.25
2000	non-Bt	7	6.5 \pm 5.3	46.6 \pm 4.3
	Bt	4	5.1 \pm 4.2	54.3 \pm 3.5

^aTable adapted from Abbas et al.⁷⁶ Results are mean of four replicates \pm standard error. ^bLevels of fumonisin in Bt hybrids were significantly lower compared to non-Bt hybrids in 1999.

hybrids of similar genetic background was assessed in USDA-ARS field trials conducted at the Mississippi State Experimental farm in 2000 and 2001.⁹² Two hybrids expressed the Syngenta Bt11 genotype (N79-L3 and N6800Bt) and three expressed the MON810 genotype (Table 6). These field trials examined the

Table 6. Aflatoxin Levels in Five (Three Isogenic and Two Non-isogenic) Bt Hybrids and Their Corresponding Non-Bt Hybrids at Mississippi State^a

		aflatoxin in harvested kernels ($\mu\text{g kg}^{-1}$)			
		2000		2001	
Bt	non-Bt	Bt	non-Bt	Bt	non-Bt
Naturally Infected					
N79-L3 ^b	N79-P4	198 ^{c*}	613	23	18
N6600Bt ^b	N6600	39 [*]	153	3	7
31B13BT ^d	3223	19 [*]	389	2	10
33 V06BT ^d	DK679	409	323	5 [*]	39
DK679BTY ^d	DK679	329	323	44	96
Inoculated with <i>Aspergillus flavus</i> and SWCB					
N79-L3 ^b	N79-P4	536 [*]	1392	147 [*]	940
N6600Bt ^b	N6600	274	415	69 [*]	247
31B13BT ^d	3223	266 [*]	878	79	208
33 V06BT ^d	DK679	302 [*]	1316	88	198
DK679BTY ^d	DK679	510	1132	405	644

^aTable adapted from Williams et al.⁷⁴ ^bHybrids express Syngenta Bt11 gene. ^{c*} indicates a significant difference ($P < 0.05$) between Bt and non-Bt hybrids for a given year. ^dHybrids express Monsanto MON810 gene.

level of aflatoxins in harvested kernels under natural conditions and artificially challenged with *A. flavus*, and the ears were mechanically inoculated with southwestern corn borer (SWCB; *D. grandiosella*) larvae. Under conditions of natural *Aspergillus* infection and normal insect pressure, three Bt-expressing hybrids had significantly lower aflatoxin levels in 2000. The reductions in aflatoxins observed in these three 2000 comparisons ranged from 95 to 68%. In 2001, natural aflatoxin pressure was lower, and only one Bt hybrid had significantly lower aflatoxin contamination in harvested kernels. When maize ears were challenged with both *A. flavus* and SWCB, the levels of aflatoxin contamination were several-fold higher, but only a few hybrid comparisons showed a significant decrease in aflatoxin contamination attributed to expression of Bt toxin.

Mississippi field trials conducted between 2002 and 2004⁹³ compared six non-Bt and six Bt maize hybrids representing short-season, midseason, and full-season maturity groups planted at three planting dates. Levels of aflatoxin in harvested kernels were related to rainfall levels and differed among years, with the highest aflatoxin level in 2002 (lowest rainfall) and the lowest aflatoxin levels in 2004 (highest rainfall). A significant reduction in aflatoxin level was observed in Bt maize compared to non-Bt (12.4 $\mu\text{g g}^{-1}$ in Bt grain compared to 45.3 $\mu\text{g g}^{-1}$ in non-Bt grain) only in 2003, with intermediate levels of aflatoxin and moderately conducive conditions. Fumonisin levels in harvested maize kernels were more consistent than aflatoxin levels in all years, with an average concentration of 5.2 mg kg^{-1} in Bt grain compared to 8.5 mg kg^{-1} in non-Bt grain ($P < 0.05$).

Initial field trials using a randomized complete block design with five replicates were conducted in Elizabeth, Mississippi, in 2006⁴⁸ to evaluate the effect of the Bt *cry1Ab* gene expressed in MON810 (Pioneer 34B24) compared to its conventional non-Bt near-isoline (Pioneer 34B23). Aflatoxin contamination of harvested kernels was significantly ($P < 0.043$) reduced by 48% in the Bt hybrid compared to its non-Bt isolate (Table 7).

Table 7. Mycotoxin Levels in Maize Expressing the *cry1Ab* Bt Gene and Its Non-Bt Isoline in Replicated Field Trials in Elizabeth, Mississippi, in 2006^a

mycotoxin	Bt	non-Bt	P
aflatoxin ($\mu\text{g kg}^{-1}$)	109 \pm 32	211 \pm 38	0.043
fumonisin (mg kg^{-1})	1.6 \pm 0.2	3.5 \pm 0.8	0.068
zearalenones (mg kg^{-1})	<0.1	<0.1	
cyclopiazaonic acid ($\mu\text{g kg}^{-1}$)	61 \pm 14	73 \pm 19	0.476
trichothecenes (mg kg^{-1})	<0.1	<0.1	

^aTable adapted from Abbas et al.³³ Results are the mean of five replicates \pm standard error.

Fumonisin contamination was reduced by 54% in the Bt compared to non-Bt hybrids, but the significance was only $P < 0.068$. A similar level of CPA was observed in both hybrids, and no deoxynivalenol or zearalenone was observed in any samples. The dominant insect pressure observed was from corn earworm, and a similar level of *A. flavus* propagules ($\log_{10} = 4.9$ colony forming units g^{-1} grain) colonized the kernels of both hybrids.

RECENT RESULTS FROM MISSISSIPPI FIELD TRIALS

Mississippi field trials discussed above were continued until 2009 and managed under no-tillage (NT) conditions with a complementary experiment initiated in 2007 that was managed under conventional tillage (CT). Conventional tillage plots were disk-harrowed in the fall following maize harvest and in the spring prior to planting. Aflatoxin and fumonisin concentrations in the grain were analyzed by LC-MS-MS and HPLC, respectively.^{94,95} Although these plots received supplemental irrigation, relatively late planting and continuous maize cultivation resulted in intensified aflatoxin and fumonisin pressure compared to that observed in 2006. It should be noted that these crop management practices were imposed to achieve a high probability of mycotoxin contamination, and not what growers would typically implement. A rather large variation of both mycotoxins was observed (Table 8). In the 2008 and 2009 NT trials and 2009 CT trials, the average concentration in Bt maize was 57–68% lower, but not statistically significant. Considering fumonisin, average levels observed in Bt maize

Table 8. Aflatoxin and Fumonisin Levels Observed in Maize Expressing the *cry1Ab* Bt Gene and Its Non-Bt Isoline in Replicated Field Trials in Elizabeth, Mississippi, Conducted under No-Till (NT) and Conventional Tillage (CT) in 2008 and 2009

year	tillage	Bt	non-Bt	P
Aflatoxin^a ($\mu\text{g kg}^{-1}$)				
2008	NT	775 \pm 777	2381 \pm 2929	0.18
	CT	272 \pm 149	266 \pm 68	0.90
2009	NT	631 \pm 537	1457 \pm 1501	0.58
	CT	755 \pm 776	2381 \pm 2926	0.18
Fumonisin^a (mg kg⁻¹)				
2008	NT	11.3 \pm 4.9	8.9 \pm 3.2	0.34
	CT	6.2 \pm 5.8	3.7 \pm 3.2	0.47
2009	NT	41.3 \pm 21.9	44.1 \pm 18.1	0.76
	CT	34.8 \pm 9.2	29.2 \pm 5.7	0.24

^aResults are the mean of five replicates \pm standard error.

were numerically greater in three of the four sets of observations. All samples contained >20 ppm regulatory allowance for aflatoxin, and all samples except for 2008 CT trials exceeded regulatory allowances for fumonisin. Thus, under conditions of high disease pressure the contribution of the Bt gene expression was not able to significantly reduce mycotoxin to a level safe for direct human consumption.

In the Mississippi trials a significant incidence of infection of grain by common smut (*Ustilago maydis*) was observed (~18% in 2006 and ~4% in 2007). From each block, 40 ears were hand-harvested that had visible smut galls as were 40 ears that showed no evidence of infection by the smut fungus. The ears were shelled, ground, and analyzed for aflatoxin and fumonisin as previously described. A significantly greater ($P < 0.05$) aflatoxin level was observed in the Bt hybrid in 2007, whereas there was no effect of hybrid on fumonisin contamination (Table 9). Significantly greater aflatoxin and fumonisin levels ($P < 0.001$) were found in grain from ears infected with smut

compared to grain from ears not exhibiting galls. Greater than 60% of samples from smut-free ears had less than the regulatory levels of fumonisin contamination. This novel observation suggests the occurrence of a fungal interaction in mycotoxin contamination, in which smut galls can allow easier ingress of earworms and airborne mycotoxin-producing fungal contaminants.

Eight commercially available Bt maize hybrids with multiple different Bt genes ("stacked gene" hybrids) (Table 10) grown in irrigated field experiments in Mississippi from 2010 to 2012 were evaluated for aflatoxin and fumonisin contamination (Bruns and Abbas, unpublished data). Two rows from each plot were treated at the V10 (10 leaf) stage of development with toxigenic *A. flavus* KS4 grown on wheat and inoculated at a rate of 22 kg ha⁻¹. Grain samples were collected at harvest from both the inoculated and untreated rows. Unpublished preliminary data from 2010 and 2011 do not show significant differences in aflatoxin and fumonisin contamination levels among the hybrids.

Most studies reviewed in this presentation have been conducted on maize hybrids with a single Bt gene. Insufficient data are available to thoroughly assess the implications of multiple (stacked) Bt genes on mycotoxin contamination. George and Crickmore¹ suggested that Bt transgenic crops can be enhanced with other strategies to broaden the insect resistance of the crop. With respect to controlling mycotoxin contamination, a potential strategy, which needs a comprehensive assessment,^{31,96,97} is to combine Bt technology with biological control using non-toxicogenic *A. flavus* isolates, because they work by different mechanisms. This combined with the economic benefit of increased yield due to reduced insect feeding^{12,52,57,58,78} provides a tremendous benefit to growers.

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Table 9. Aflatoxin and Fumonisin Contamination of Symptomless Maize Kernels in Elizabeth, Mississippi, Field Trials: Interactions of Bt Gene and Infection by Smut^a

hybrid	smut	aflatoxin ($\mu\text{g kg}^{-1}$)		fumonisin (mg kg ⁻¹)	
		2006	2007	2006	2007
Bt	none	39 \pm 42	0.5 \pm 0.5	3 \pm 1	5 \pm 3
Bt	infected	96 \pm 51	183 \pm 347	180 \pm 153	176 \pm 117
non-Bt	none	67 \pm 78	33 \pm 55	4 \pm 2	2 \pm 1
non-Bt	infected	2211 \pm 1787	1889 \pm 2252	212 \pm 24	121 \pm 83

^aResults are the mean of five replicates \pm standard error.

Table 10. Characteristics of Maize Hybrids Evaluated for Mycotoxin Contamination in 2010–2012

hybrid	trait branding ^a	foreign genes expressed ^b	herbicide tolerance ^c	transformation event
DKC 66-96	Genuity VT Triple PRO	(Cry1A.105, Cry2Ab) + (CP4 EPSPS, Cry3Bb)	glyphosate	Mon88017 + Mon89034
DKC 67-21	Genuity VT Triple PRO	(Cry1A.105, Cry2Ab) + (CP4 EPSPS, Cry3Bb)	glyphosate	Mon88017 + Mon89034
DKC 67-22	RoundUp Ready (RR) 2	CP4 EPSPS	glyphosate	GA21
31G96	Herculex, LL, RR2	CP4 EPSPS + (PAT, Cry1F)	glyphosate, glufosinate	TC1507
31P42	Herculex, LL, RR2	CP4 EPSPS + (PAT, Cry1F)	glyphosate, glufosinate	TC1507
1615R	RR2	CP4 EPSPS	glyphosate	GA21
31P40	RR2	CP4 EPSPS	glyphosate	GA21
33N55	RR2	CP4 EPSPS	glyphosate	GA21

^aRoundUp Ready 2, VT Triple Pro, and Genuity are trademarks of Monsanto. Herculex is a trademark of Dow Agroscience. ^bGenes listed within parentheses are a single transformation event. ^cGlyphosate tolerance allows in-season application of herbicides such as RoundUp, Touchdown, and Accord. Glufosinate tolerance allows in-season application of herbicides such as Liberty.

Notes

The authors declare no competing financial interest.

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