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Investigating Transgenic Corn Hybrids as a Method for Mycotoxin Control

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Abstract

Transgenic Bt corn hybrids have been available for more than 10 years and are known to control specific insects. More recently, so-called "stacked-gene" hybrids, have been released with multiple insect resistance genes and genes for herbicide resistance, resulting in up to 6 traits per plant. Because insect damage can lead to increased levels of mycotoxins, such as aflatoxins and fumonisin, we designed a study to compare ten commercially available corn hybrids, two non-transgenic, four with both herbicide and insect tolerance (stacked-gene) and four with glyphosate tolerance only to determine if any hybrid class had the advantage of reduced mycotoxin contamination. The experiment was carried out in the Mississippi State University Delta Research Extension fields in Stoneville, MS for two years in fine sandy loam and clay soil. Rows were either inoculated at the V10 stage of growth with toxigenic *Aspergillus flavus* K54 (NRRL 58987, isolated from corn kernels in Mississippi), grown on wheat, and applied at a rate of 22.42 kg/ha or allowed to become naturally infected with disease-producing fungi, including various *Fusarium* and other *Aspergillus* spp. Mycotoxin production differed according to the soil type with lower levels detected in the hybrids planted in clay soil vs. sandy soil. However, no significant differences in mycotoxin production were found amongst the hybrid classes. More research is needed to identify conditions under which transgenic hybrids might produce higher yields and lower mycotoxin levels. Presently, selection of transgenic hybrids will not replace integrated strategies of biocontrol, host plant resistance, or good crop management practices for achieving adequate mycotoxin control in corn.

Keywords

Stacked-Gene Corn, Hybrids, Soil Type, Mycotoxins, Aflatoxin, Fumonisin

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1. Introduction

The use of transgenic crops has become increasingly common over the past 10 years in the U.S. and some areas around the world [1]. Most of these transgenic crops such as corn, soybean, and cotton, were generated to manage agricultural pests including insects, weeds, and to some extent diseases. Initially, transgenics started with a single trait to control a specific pest [2]-[11]. Recently, new technology has emerged and generated crops with multiple engineered traits which are called "stacked-gene hybrids" enabling them to control more than one agricultural pest [12] [13]. Evaluation of transgenic corn hybrids in regard to mycotoxin management has not been well established, especially in regard to aflatoxin and fumonisin. While Bt corn has demonstrated some mediating effect on fumonisin, aflatoxin, and trichocenes, no transgenic hybrids have been developed specifically to lower mycotoxin contamination [1] [14]-[16]. The increased level of mycotoxins, due to insect damage, was thought to be due to the fact that insect feeding allows fungi to enter the plant and produce mycotoxins [17]-[20]. Research has shown that fumonisin levels are reduced in Bt corn, but no conclusions could be drawn about possible reductions in aflatoxins [1]. Corn contaminated with mycotoxins may lead to major food safety issues, production losses and grain waste [21]. Aflatoxins are a group of chemical compounds produced primarily by *Aspergillus flavus*, as well as other *Aspergillus* spp. Aflatoxins and fumonisins can both be serious problems in the southern U.S., while fumonisins are often more prevalent in more northerly areas of the U.S. [22]-[24].

A number of environmental parameters affect mycotoxin production, including heat, drought, insect infestation and various plant diseases [25]-[31]. When both toxins are found together, the risk of toxicity increases [24] [32]-[34]. Theoretically, stacked-gene hybrids should be beneficial to reduce mycotoxins, as they are formulated to deal with factors affecting mycotoxin production. The objective of this study was to investigate the effect of stacked-gene corn hybrids on the accumulation of fumonisins and aflatoxins, compared to single trait glyphosate resistant and non-transgenic hybrids under irrigated field conditions in the Mississippi Delta, and was part of an experiment previously reporting the yield and economics of three classes of corn hybrids [12].

2. Materials and Methods

2.1. Stacked-Gene Hybrids and Field Conditions

Ten corn hybrids, four stacked-gene, four glyphosate-tolerant and two non-GMO, were purchased commercially in 2011, and were used in both 2011 and 2012 (**Table 1**). Between planting seasons, remaining seed were preserved in cold storage (4˚C). The research was conducted at two sites, one a Bosket fine sandy loam (fine-loamy, mixed, active, thermic Mollic Hapludalfs), located on the Mississippi State University Delta Branch Research

Table 1. Conventional and transgenic corn hybrids used in 2011 and 2012 study.

Hybrid[†]	Trait branding	Insect traits	Herbicide tolerance	Transformation event
31P41		None	None	
33N56		None	None	
1615R	RR2	None	Glyphosate	
31P40	RR2	None	Glyphosate	
33N55	RR2	None	Glyphosate	
DKC 67-22	RR2	None	Glyphosate	
31G96	HX1, LL, RR2	Cry1 F	Glyphosate, glufosinate	TC1507
31P42	HX1, LL, RR2	Cry1 F	Glyphosate, glufosinate	TC1507
DKC 66-96	Genuity VT Triple PRO	Cry1A.105, Cry2 Ab2, Cry3 Bb	Glyphosate	Mon88017 + Mon89034
DKC 67-21	Genuity VT Triple PRO	Cry1A.105, Cry2 Ab2, Cry3 Bb	Glyphosate	Mon88017 + Mon89034

[†]31P41 and 33N56 are conventional hybrids; 1615R, 31P40, 33N55, and DKC 67-22 are single trait glyphosate tolerant only hybrids with no Bt; and 31G96, 31P42, DKC 66-96, and DKC 67-21are classed as Stacked-Gene Hybrids with insect and herbicide resistance traits.

and Extension Center, Stoneville, MS, and the other a Tunica clay (clayey over loamy, smectitic, nonacid, thermic Vertic Haplaquept) on private property 1.5 k north of Elisabeth, MS, U.S.A. Each hybrid was planted in eight row plots (1.4 meter between rows 12 meter long) with a final stand density of approximately 31,000 kernels·A^{-1}. The experiments were planted 7 April 2011 and 29 March, 2012. All experiments were conducted in conventional tillage plots, furrow irrigation was performed, and all other agronomic measurements as previously described by Bruns [12]. Rows 7 and 8 within each plot were inoculated at the V10 stage of growth with toxigenic *Aspergillus flavus* K54 (NRRL 58987, isolated from corn kernels in Mississippi), grown on wheat, and applied at a rate of 22.42 kg/ha [35]. The remaining 6 rows were allowed to become naturally infected with disease-producing fungi, including various *Fusarium* and other *Aspergillus* spp.

The mature crop was harvested with a two row combine. The non-inoculated rows were harvested first, and then the inoculated rows were harvested. Two different combines were used; one to harvest the inoculated rows (Gleaner K2, AGCO, Duluth, GA) and the other (Kincaid, Haven, KS) to harvest the non-inoculated rows to prevent cross-contamination. Yield and yield components were determined and previously published [12].

2.2. Mycotoxin Analysis

Sub-samples of 2 kg of kernels from each treatment were pooled, mixed, oven dried at 50˚C for 3 to 5 d, and ground (20 mesh) using a Romer mill (Union, MO). A representative 50 g sub-sample was taken and extracted in 250 mL of 70% methanol as previously described [23]. Both aflatoxin and fumonisin levels were determined by High-Pressure Liquid Chromatography (HPLC) with post-column derivations as described below.

2.3. Aflatoxin Determination

Preparation, cleanup and determination of aflatoxin in samples were performed according to protocols as described in detail previously by Abbas *et al.* [23]. Briefly, aflatoxin levels were analyzed by WATERS HPLC with post-column photochemical derivation (PHRED) (Aura Industries, New York, NY) and fluorescence detection, containing a WATERS 717 Autosampler. Detection limit of this procedure was 0.1 ng/g, and the standard curve was linear up to 200 ng/g.

2.4. Fumonisin Determination

Fumonison samples extracted in 70% methanol were cleaned up using Bond-Elute SAS columns (Varian, Harbor City, CA) [23] [25] [36]. The columns were washed using 2.5 mL each of 100% methanol and 75% methanol. Next, 2.5 mL of the sample extract was applied to the column, and washed again with 75% methanol and 100% methanol applied to the column. The sample was eluted using 2.5 mL of 2% acetic acid in methanol, dried under nitrogen at 50˚C using a Turbo Vap LV (Biotage, Charlotte, NC), and stored at −20˚C until ready for analysis. The clean samples were reconstituted in 1 mL acetonitrile: water (30:70) and analyzed by HPLC with post-column derivatization according to the method described in [37], Joerg Stroke, Institute for Reference Materials and Measurements, Belgium (Personal communication)] with little modification. Samples to be analyzed for fumonisin were injected onto an Agilent 1200 system consisting of a binary pump, autosampler, and a fluorescence detector equipped with a 150 mm × 4.6 mm i.d. 5 µm Zorbax Eclipse XDB-C18 column at a temperature of 45˚C. A secondary pump (Waters Reagent Manager) was also attached to the system connected in-line after the column and before the detector. This allowed for mixing of the injected sample and a derivatization solution before going to the detector. The detector was set at 335 nm (excitation) and 440 nm (emission). The mobile phases for this system were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) in a gradient at a flow rate of 1.2 mL/min. for 18 minutes beginning at 68% A and 32% B for 8 minutes, switching to 60% A and 40% B for 3 minutes, and then back to 68% A and 32% B for the rest of the injection. The post-column reaction solution was prepared using sodium carbonate, boric acid, potassium sulfate, N-Acetyl-L-Cysteine, and o-phthaldialdehyde (OPA) and run at a constant flow rate of 0.45 mL/min. Standards for this analysis were prepared using fumonisin B_1 and B_2 (Sigma products F1147 and F3771, respectively) in 30% acetonitrile in water. The 30% acetonitrile was used as blanks for the analysis. The limit of quantitation was 0.025 ng/µL.

2.5. Experimental Design and Statistical Analysis

The experiment was a randomized complete block design with four replicates for each hybrid. Analyses of va-

riance were conducted using Proc mixed of SAS (9.7) (SAS Institute, Cary, NC). Year, soil, cultivar, and genetic background were considered fixed effects. Replicate (Rep) and Rep (Year) were considered random effects. Residual values shown in **Table 5** and **Table 6** refer to Restricted Maximum Residual Likelihood (REML), which reflects the total variance of the random parameters in the model.

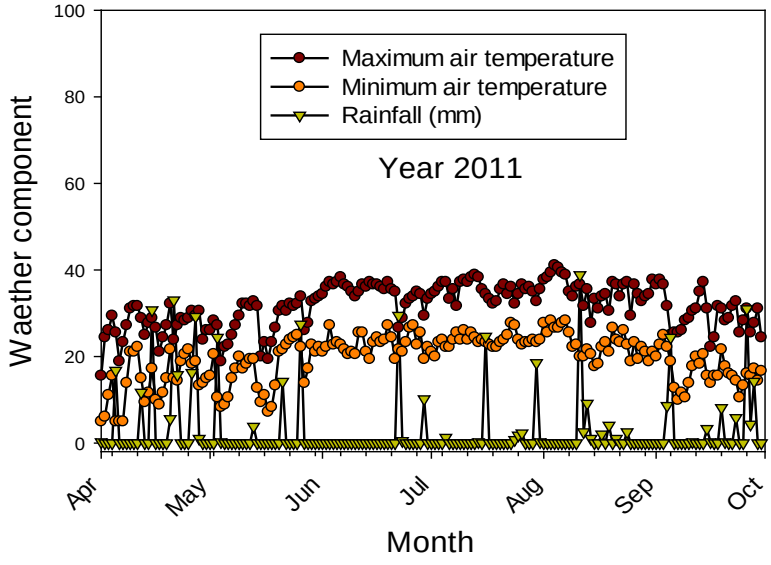
3. Results and Discussion

Hybrid had no significant effect on aflatoxin, but interaction with year (hybrid × year) or year × soil × hybrid was statistically significant (**Table 2**). Inoculation had significant effects for aflatoxin, but its interactions with the rest of factors (year, soil, and hybrid) did not show significant effects. Although the response of fumonosin was similar to aflatoxin for some factors such as the effects of year, soil, and their interactions (**Table 2**), fumonisin responded differently to other factors in that cultivar, year × soil × cultivar, soil × Inoc, and year × cultivar × Inoc had significant effects (**Table 2**). The significant effects of year, soil, and their interactions indicated the significant of the environmental factors such as heat, drought, and soil on toxins. The weather data [38] revealed that year 2011 was warmer and drier compared with year 2012 (**Figure 1(a)** and **Figure 1(b)**), especially during the reproductive and seed-fill stages. This could be a source of effects on inoculation. The different response of aflatoxin and fumonisin to some factors and their interactions reflects the different sensitivity of each toxin to these factors. Both toxins were significantly influenced by the inoculation, indicating that the level of these toxins in the plant is affected by the disease infection. To further evaluate the effects of the three genetic backgrounds (stacked gene hybrids with multiple Bt genes; Round-up Ready, RR2; and non-transgenic) on aflatoxin and fumonisin levels in seeds, data were analyzed where genetic background was modeled with year, soil, and inoculation (**Table 3**). The analysis confirmed that year, soil, and their interactions, and Inoc had significant influences on aflatoxin and fumonisin levels. However, genetic background or its interactions had no significant influences on either aflatoxin or fumonisin levels, indicating that inoculation influenced the levels of aflatoxin and fumonisin, but the inoculation effects did not depend on the genetic background.

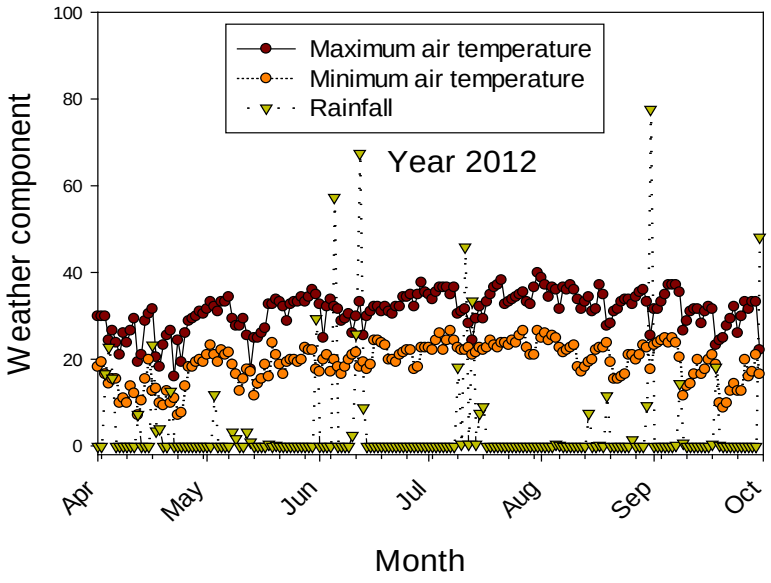
Table 2. Analysis of variance (F values and P values) for the effects of year, cultivar, soil, and inoculation (Inoc), and their interactions on aflatoxin (μg/kg) and fumonisin (mg/kg) in corn cultivars (stacked gene hybrids and control) in sandy and clay soils under Mississippi delta condition.

Effect	Num DF	Aflatoxin		Fumonisin	
		F Calc.	P	F Calc.	P
Year	1	33.69	***	20.70	***
Soil	1	50.33	***	4.93	*
Year × Soil	1	70.07	***	18.74	***
Cultivar	9	0.88	NS	5.98	***
Year × Cultivar	9	4.13	***	4.16	***
Soil × Cultivar	9	3.44	***	1.25	NS
Year × Soil × Cultivar	9	1.48	NS	2.20	*
Inoc	1	8.57	**	12.85	***
Year × Inoc	1	0.31	NS	0.08	NS
Soil × Inoc	1	0.79	NS	10.64	***
Year × Soil × Inoc	1	3.09	NS	63.68	***
Cultivar × Inoc	9	0.45	NS	1.35	NS
Year × Cultivar × Inoc	9	0.97	NS	0.56	NS
Soil × Cultivar × Inoc	9	0.71	NS	0.77	NS
Year × Soil × Cultivar × Inoc	8	0.73	NS	1.33	NS
Rep (Year)		0		0	
Residual		23659		4.91	

*Significance at $P \leq 0.05$; **Significance at $P \leq 0.01$; ***Significance at $P \leq 0.001$.



(a)



(b)

Figure 1. Weather data for 2011 and 2012 in the Mississippi Delta.

3.1. Levels of Aflatoxin and Fumonisin in Hybrids

The levels of aflatoxin in samples from the Tunica clay compared to Bosket FSL are shown in **Table 4**. Aflatoxin levels observed among samples of the non-inoculated corn of the ten hybrids from the Tunica clay type was variable ranging from 1.4 µg/kg for stacked corn hybrid 31G96 to 88.9 µg/kg for stacked corn hybrid DK67-21. Aflatoxin levels among the inoculated corn of the same ten hybrids were variable as well ranging from 0.1 µg/kg for RR2 corn hybrid 31P40 to 207.4 µg/kg for RR2 corn hybrid DKC67-22. Overall aflatoxin levels in the ten corn hybrids tested were higher from inoculated soils than from non-inoculated soils, and varied significantly ($P \leq 0.05$) among the ten corn hybrids tested in the inoculated fields in comparison to the levels of aflatoxin among the same hybrids tested in the non-inoculated soil. Levels of aflatoxin were variable and no one hybrid or group of hybrids was consistent in the levels of aflatoxin contamination when grown in Tunica clay under all experimental conditions.

Table 3. Analysis of variance (F values and P values) for the effects of year, cultivar, soil, and inoculation (Inoc) on aflatoxin (µg/kg) and fumonisin (mg/kg) on corn genetic background [(stacked gene hybrids with multiple Bt genes; Round-up Reddy, RR2; and non-GMO)] in sandy and clay soils under Mississippi delta conditions.

Source effects	DF	Aflatoxin		Fumonisin	
		F-Calc.	P	FCalc.	P
Year	1	32.27	***	12.91	***
Soil	1	43.4	***	2.88	*
Year × Soil	1	59.17	***	9.91	**
Genetic background	2	0.29	NS	1.27	NS
Year × Genetic background	2	2.16	NS	0.73	NS
Soil × Genetic background	2	0.3	NS	0.32	NS
Year × Soil × Genetic background	2	2.38	NS	1.03	NS
Inoc	1	7.03	**	7.95	**
Year × Inoc	1	0.01	NS	0.07	NS
Soil × Inoc	1	1.06	NS	9.43	**
Year × Soil × Inoc	1	3.83	*	45.3	***
Genetic background × Inoc	2	0.17	NS	0.14	NS
Year × Genetic background × Inoc	2	1.25	NS	0.61	NS
Soil × Genetic background × Inoc	2	0.2	NS	0.13	NS
Year × Soil × Genetic background × Inoc	2	0.56	NS	0.2	NS
Rep (Year)		1.24E-14		0	
Residual		27183		6.4579	

*Significance at $P \leq 0.05$; **Significance at $P \leq 0.01$; ***Significance at $P \leq 0.001$.

Table 4. Aflatoxin contamination levels of ten irrigated corn hybrids representing three genotype classes grown on two sites, a Tunica Clay and a Bosket Fine Sandy Loam (FSL) near Stoneville, MS across 2011 and 2012[†].

Hybrid	Hybrid class	Aflatoxin (µg/kg)			
		Tunica clay		Bosket FSL	
		Non-Inc[‡]	Inc[§]	Non-Inc[§]	Inc[§]
31P41	Non	14.6	21.5b	149.6b	154.9ab
33N56	Non	14.8	30.2b	91.6b	267ab
1615R	RR2	12.2	18.3b	115.4b	143.3ab
31P40	RR2	4.9	0.1b	125.7b	329.9ab
33N55	RR2	6.9	44.8b	129.4b	158.2ab
DKC 67-22	RR2	7.4	207.4a	59.3b	118b
31G96	Stacked	1.4	22.9b	289.4a	335.9a
31P42	Stacked	2.9	16.2b	136.8b	153.5ab
DKC 66-96	Stacked	5	36.2b	114.2b	176.2ab
DKC 67-21	Stacked	88.9	153.8ab	43.1b	93.4c

[†]Means of 4 replications and 2 years. [‡]Means are not significantly different. [§]Means within a column followed by the same letter or letters are not significantly different $P \leq 0.05$.

Aflatoxin levels of corn grown in Bosket FSL soil were higher ranging from 43.1 µg/kg for stacked corn hybrid DKC67-21 to 335.9 µg/kg for the stacked corn hybrid 31G96 under both non-inoculated and inoculated conditions compared with the overall levels of aflatoxin in Tunica clay soil (**Table 4**). Although there were significant differences ($P \leq 0.05$) in aflatoxin levels among hybrids in Bosket FSL soil, all hybrids had aflatoxins above the regulatory level of 20 µg/kg.

The levels of fumonisin of the combined samples harvested in 2011 and 2012 are shown in **Table 5** Fumonisin levels were less variable overall than aflatoxin levels ranging from 0.5 to 16.2 mg/kg. Average fumonisin levels were about the same in all hybrids in both non-inoculated and inoculated fields both years (**Table 5**), and differences were highly significant ($P \leq 0.05$) among all hybrids. Fumonisin levels among all hybrids grown in non-inoculated and inoculated Bosket FSL soil type were not significantly different ranging from 1.2 mg/kg to 5.0 mg/kg (**Table 5**). Observations of fumonisin levels among the hybrids were similar to observations of aflatoxin levels among the hybrids. No single hybrid or group of hybrids stood out as resistant or susceptible.

Aflatoxin levels were quite different between 2011 and 2012, possibly due to temperature and rainfall conditions despite irrigation. Differences in aflatoxin levels were evident between 2011 and 2012 in grain samples from non-inoculated or inoculated treatments in Bosket FSL soil. The aflatoxin levels in the 2011 samples ranged from 237.5 to 339.9 µg/kg for non-inoculated and inoculated Bosket FSL soil, respectively, and were higher than aflatoxin levels from corn samples from the Tunica Clay soil (**Table 6**). Likewise, aflatoxin levels in the 2012 samples were lower than in 2011 samples and ranged from 11.8 µg/ kg to 52.1 µg/kg for the non-inoculated and inoculated field, respectively. The aflatoxin levels in harvested corn samples in 2011 and 2012 were lower in the non-inoculated Tunica Clay field, ranging from 15.2 µg/kg in 2012 to 15.9 µg/kg in 2011.

Fumonisin levels in corn samples harvested from Tunica Clay and Bosket FSL soil types were inconsistent as far as non-inoculated and inoculated fields in 2011 and 2012 (**Table 6**). Fumonisin levels in samples from both non-inoculated and inoculated soils in the Tunica clay field in 2011 were almost identical at 2.3 and 2.2 mg/kg, respectively. In 2012, the levels were significantly different at 0.3 and 4 mg/kg, respectively ($P \leq 0.05$). Samples harvested from the non-inoculated and inoculated Bosket FSL fields were 2.9 and 6.0 mg/kg in 2011 and 2.6 and 0.6 mg/kg in 2012. Yields of the various corn hybrids used in this study were higher for all hybrids in the Tunica clay soil [12] compared to the same hybrids in the sandy loam Bosket FSL. During the course of the experiments, hybrids in the Tunica clay soil exhibited better plant growth and health (Bruns, Unpublished data).

Table 5. Fumonisin contamination levels of ten irrigated corn hybrids representing three genotype classes grown on two sites, a Tunica Clay and a Bosket Fine Sandy Loam (FSL) near Stoneville, MS across 2011 and 2012[†].

Hybrid	Class	Fumonisin (mg/kg)			
		Tunica clay		Bosket FSL	
		Non-Inc[§]	Inc[§]	Non-Inc[§]	Inc[‡]
31P41	Non	1.1bc	1.4c	2.3	4.4
33N56	Non	2.0b	5.7a	3.6	5
1615R	RR2	0.6c	2.1bc	2.3	2.5
31P40	RR2	1.9b	1.6c	2.9	3.2
33N55	RR2	1.2b	3.7b	3.9	4.3
DKC 67-22	RR2	0.5c	2.3bc	1.4	2.4
31G96	Stacked	2.4b	5.9a	3.1	2.4
31P42	Stacked	16.2a	2.4bc	3.7	2.7
DKC 66-96	Stacked	1.8b	5.7a	2.4	1.2
DKC 67-21	Stacked	0.5c	2.4bc	1.8	2.1

[†]Means of 4 replications and 2 years; [‡]Means are not significantly different; [§]Means within a column followed by the same letter or letters are not significantly different $P \leq 0.05$.

Table 6. Aflatoxin and Fumonisin contamination levels of irrigated corn hybrids grown on two sites, a Tunica Clay and a Bosket Fine Sandy Loam (FSL) Near Stoneville, MS across 2011 and 2012[†].

	Aflatoxin (µg/kg)			
	Tunica clay		Bosket FSL	
Year	Non-Inc[‡]	Inc[§]	Non-Inc[§]	Inc[§]
2011	15.9	7.6	237.5	339.9
2012	15.2	92.9	11.8	52.1
	Fumonisin (mg/kg)			
	Tunica clay		Bosket FSL	
Year	Non-Inc[§]	Inc[§]	Non-Inc[‡]	Inc[§]
2011	2.3	2.2	2.9	6.0
2012	0.3	4	2.6	0.6

[†]Means of 10 hybrids representing 3 genotypes and 4 replications. [‡]Means are not significantly different. [§]Means within a column are significantly different $P \leq 0.05$.

The theory that reducing plant stress during the kernel-filling time will cause reductions in mycotoxin levels was tested in this research by evaluating if weather data during the 2011 and 2012 growing seasons might correlate with aflatoxin and fumonisin levels (**Figure 1**). Weather comparisons between the 2 years were significantly different in rainfall. For example, the maximum temperature in June, July, and August 2011 were 34.9, 35.4, and 35.4˚C, respectively, compared to 31.7˚C, 33.7˚C, and 34.1˚C in 2012 (**Figure 1**). Similar trends for rainfall were observed where the precipitation was lower during June, July, and August, resulting in a warmer and drier year in 2011 than in 2012, although the precipitation was higher early in the season in 2011 compared to 2012. These conditions may have created additional stress due to warmer and drier season, especially during the kernel-filling stage and contributed to much higher aflatoxin contamination in sandy soils. Field water holding capacity of sandy soils is lower than that of clay soils, consequently crops grown in sandy soil are more sensitive to drought stress even when the irrigation is used. Fumonisin levels in the corn samples did not consistently relate to weather conditions. Recently it was found that these hybrids did not yield as well in sandy soil, possibly due to drought stress even when they received adequate irrigation, (Bruns, unpublished data) compared to the same hybrids grown in clay soil [12] [25] [39]. In all cases corn grown in sandy loam soil had much higher levels of aflatoxin. None of the hybrids, conventional or stacked-gene, were clearly superior to the others in terms of aflatoxin levels. Some reports of hybrids with a Bt gene showed variable effects on mycotoxin levels. Some authors reported beneficial effects of hybrids, others did not [1] [40]-[43]. A multifaceted strategy to reduce mycotoxins appears to be necessary [44].

4. Conclusion

It is not clear at this time whether the benefits of stacked-gene hybrids justify the costs of their development [12]. To reach a conclusive recommendation, more research is required where more hybrids are tested across more years, geographic locations, and under irrigated and non-irrigated conditions.

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