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Aflatoxin and Fumonisin Contamination of Commercial Corn (*Zea mays*) Hybrids in Mississippi

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Resistance to mycotoxin contamination was compared in field samples harvested from 45 commercial corn (maize) hybrids and 5 single-cross aflatoxin-resistant germplasm lines in years with high and moderate heat stress. In high heat stress, mycotoxin levels were (4.34 ± 0.32) × 10^3 μg/kg [(0.95−10.5 × 10^3 μg/kg] aflatoxins and 11.2 ± 1.2 mg/kg (0−35 mg/kg) fumonisins in commercial hybrids and 370 ± 88 μg/kg (140−609 μg/kg) aflatoxins and 4.0 ± 1.3 mg/kg (1.7−7.8 mg/kg) fumonisins in aflatoxin-resistant germplasm lines. Deoxynivalenol was detected (one-fourth of the samples, 0−1.5 mg/kg), but not zearalenone. In moderate heat stress, mycotoxin levels were 6.2 ± 1.6 μg/kg (0−30.4 μg/kg) aflatoxins and 2.5 ± 0.2 mg/kg (0.5−4.8 mg/kg) fumonisins in commercial hybrids and 1.6 ± 0.7 μg/kg (0−7 μg/kg) aflatoxins and 1.2 ± 0.2 mg/kg (0.5−3.0 mg/kg) fumonisins in aflatoxin-resistant germplasm lines. The results are consistent with heat stress playing an important role in the susceptibility of corn to both aflatoxin and fumonisin contamination, with significant reductions of both aflatoxins and fumonisins in aflatoxin-resistant germplasm lines.

KEYWORDS: Aflatoxin; fumonisin; corn hybrids; maize; zearalenone; deoxynivalenol; mycotoxin; heat stress; drought; aflatoxin-resistance; *Aspergillus*; *Fusarium*

INTRODUCTION

Corn (maize, *Zea mays*) is frequently infected with fungi, which produce toxins that affect the quality and safety of human food and animal feeds. Two of the most serious corn contamination problems are due to *Aspergillus flavus* and *Fusarium verticilliodes* (Sacc.) Nirenberg [syn.: *F. moniliforme*], which produce aflatoxins and fumonisins, respectively (**Figure 1**) (*1*−*4*). Aflatoxins are powerful hepatotoxins, teratogens, mutagens, and carcinogens (*5*−*7*). Aflatoxin contamination is responsible for severe economic losses in the corn crop in the South and the Midwest (*6*−*9*; W. Mubatanhema, Z. Jurjevic, D. M. Wilson, B. Evans, N. Widstrom, and F. I. Meredith, unpublished results). Fumonisins have been reported to induce several diseases in animals, notably leukoencephalomalacia in horses and pulmonary edema in swine, and they also act as tumor promotors in rats (*10*−*13*). It has been reported that *F. verticilliodes* contamination of food may be associated with esophageal cancer in humans (*14*−*16*). Recently, a 2-year study by the U.S. Food and Drug Administration (FDA) demonstrated that chronic feeding of purified fumonisin B_1 produced liver cancer and decreased life span in female mice and induced kidney cancer in male rats without decreased life span (*17*). Also, it has been reported that fumonisins induced apoptosis in cultured human cells and in rat kidneys (*18*). Carlson et al. (*19*) showed that fumonisin B_1 promoted aflatoxin B_1-initiated liver tumors in trout fed ≥23 mg/kg fumonisin B_1 for 42 weeks. The FDA has recently issued guidelines for fumonisin levels in human foods and animal feeds and recommended maximum levels of total fumonisins as low as 2 mg/kg in degermed dry-milled corn products intended for human consumption (*20*). The nutritional and economic importance of corn in the United States is balanced by the risk of a serious health hazard due to aflatoxin−fumonisin contamination. Trichothecene toxins, including deoxynivalenol and others, and non-trichothecene *Fusarium* toxins such as zearalenone have been found in grains and animal feeds (*21*−*23*). They have been shown to cause toxicological problems in farm and experimental animals (*24*−*26*).

The production of mycotoxins in corn is often influenced by factors that stress corn plants (*6*). Stress factors include moisture content of the soil, high daytime maximum temperatures, high nighttime minimum temperatures (which strongly favor fungal growth), and nutrient-deficient soils (*27*). In 1998 drought and

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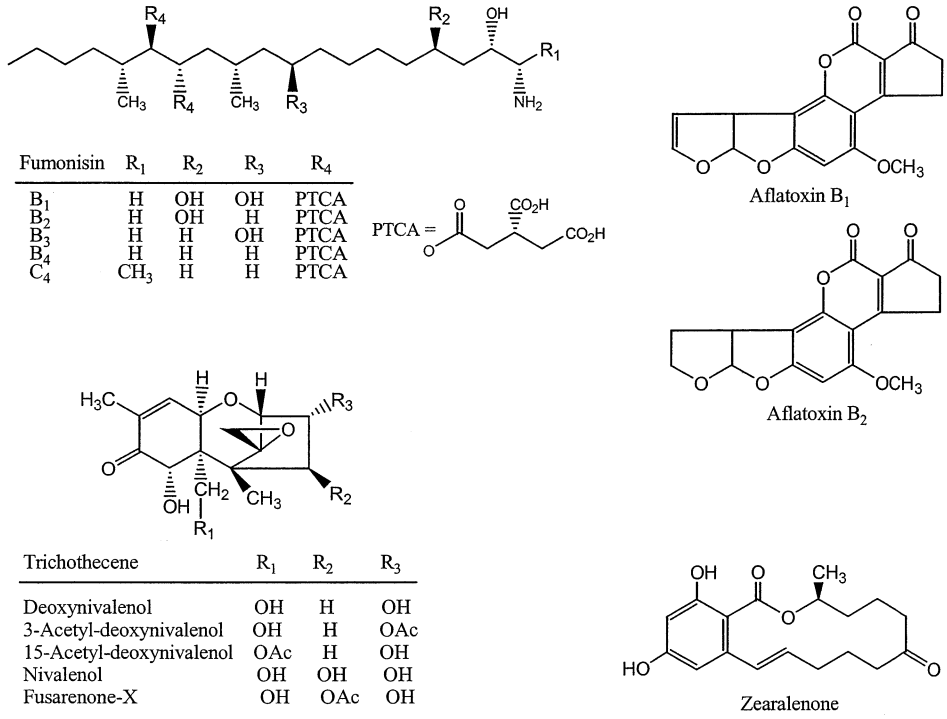

Figure 1. Structures of mycotoxins investigated.

high-temperature conditions in Mississippi were thought to contribute to high levels of corn contamination by both aflatoxin and fumonisin (*9*). The present study was conducted to investigate the effects of environmental stress on aflatoxin and fumonisin production in commercial hybrids and known resistant germplasm lines by comparing mycotoxin production in a high-stress growing season (1998) with production in a subsequent moderate-stress growing season (1999). To focus the study on the effects of environmental stress on corn plants, not on the amount of fungus present in the environment, all corn hybrids were inoculated with *A. flavus* spores in both years.

MATERIALS AND METHODS

Corn Hybrids. Forty-five commercial corn hybrids and 5 single-cross aflatoxin-resistant germplasm lines were grown at the Plant Science Research Farm, Mississippi State University, near Starkville, MS, in 1998. Twenty-two commercial corn hybrids and 12 single-cross aflatoxin-resistant germplasm lines were grown in 1999 at the Plant Science Research Farm at Mississippi State University and at the Delta Research and Extension Center, Stoneville, MS. Hybrids were planted May 4, 1998, and April 23, 1999, at Starkville, and were planted April 16, 1999, in Stoneville. Hybrids were grown in a randomized complete block design with five replications. Each plot was a single row 5.1 m in length, and seedlings were thinned to 20 per plot.

Inoculation of Corn Hybrids. In 1998, 45 commercial corn hybrids and 14 aflatoxin-resistant germplasm lines (**Table 1**) planted in Starkville, MS, were inoculated with *A. flavus* to compare their resistance to aflatoxin contamination in the field (*8*). Inoculation with *A. flavus* spores was used to ensure uniform exposure to the fungus, regardless of environmental conditions. Because *F. verticilliodes* is consistently associated with corn (*28*), it was not considered necessary to inoculate corn hybrids with *F. verticilliodes* spores.

A. flavus isolate NRRL 3357, which is known to produce aflatoxin in corn grain (*29*), was used as inoculum in all tests. The inoculum was produced on corn cob grits placed in 500 mL flasks, each containing 50 g of grits and 100 mL of water, sterilized by autoclaving, inoculated with sections of *A. flavus* culture growing on potato dextrose agar, and incubated at 28 °C for 2−3 weeks (*8*). Conidia were washed from the grits using sterile distilled water containing 20 drops of Tween 20/L and filtered through four layers of sterile cheesecloth. The concentration of conidia was determined with a hemocytometer and adjusted with sterile distilled water to 9×10^7 conidia/mL. Inoculum not immediately used was refrigerated at 4 °C. Hybrids were inoculated using a Solo backpack sprayer (Solo, Newport News, VA). A spore suspension containing *A. flavus* conidia and a spreader sticker (Hi-Yield Chemical Co., Bonham, TX) was applied to the silks and husk on the top ear of each plant. Hybrids were inoculated weekly for 5 weeks beginning when silks emerged from the ears.

Collection of Corn Samples. Ears were hand harvested about 63 days after midsilk and dried at 38 °C for 7 days. Ears were machine shelled, and grain samples (at least 1 kg) from each row were poured into a sample splitter twice to mix the grain. Samples (1 kg) were ground using a Romer mill (Union, MO). Part (one-third) of each ground sample was selected and extracted as described below.

Materials. All solvents were of HPLC grade from Fisher Scientific (Pittsburgh, PA). All mycotoxin standards (aflatoxins B_1, B_2, G_1, and G_2 and a combined standards mixture described below; fumonisins B_1 and B_2; deoxynivalenol; and zearalenone) and other chemicals were obtained from Sigma (St. Louis, MO). HPLC columns, guard columns, and the Visiprep solid phase extraction system were from Supelco (Bellefonte, PA).

Extraction of Mycotoxins from Corn Samples. Subsamples of ground field corn (20 g) were extracted with 100 mL of methanol/water, 70:30. The samples were placed on a shaker table and shaken for 3−5 min at high speed and then filtered through Whatman no. 1 filters. Ten-milliliter portions of each sample were placed in two labeled 16 × 100 mm glass test tubes for mycotoxin analyses. To minimize fumonisin side-chain hydrolysis or side-chain migration in partially hydrolyzed fumonisins, samples were evaporated to dryness in a Zymark TurboVap LV evaporator under a stream of nitrogen and stored at 5 °C until subjected to cleanup and HPLC analysis.

Aflatoxin Cleanup. Aflatoxin samples were dissolved in 10 mL of 70% methanol. Ten aflatoxin affinity columns (Neogen Corp., Lansing, MI) were set up on a Supelco 24TM DL solid phase extraction tank to clean 10 samples at a time. The columns were conditioned by passing 2 mL of 100% methanol followed by 10 mL of 25% methanol in water through them. Each 10 mL reconstituted sample was passed through a column under vacuum. Two 10 mL volumes of 25% methanol in water were passed through each sample holding column. A labeled tube was placed under each column, and 2 mL of 100% methanol was used to elute the sample. Samples were prepared for HPLC analysis, and the

Table 1. Aflatoxin Levels in Corn Samples Collected in 1998 and 1999[a]

	aflatoxins (μg/kg)				
	1998, Starkville[b]			1999, Starkville	1999, Stoneville
hybrid no.	B_1	B_2	total	total	total
			Commercial Hybrids		
1177	0.95×10^3	0.0	0.95×10^3	NT	NT
5688	3.37×10^3	T	3.37×10^3	1.8	0.9
5510A	2.14×10^3	0.14×10^3	2.27×10^3	1.8	2.4
8460	3.10×10^3	0.19×10^3	3.28×10^3	20.7	1.9
TV2930	4.86×10^3	T	4.86×10^3	NT	NT
DK683	4.36×10^3	0	4.36×10^3	T	0.0
5670	7.83×10^3	T	7.83×10^3	5.6	1.9
TR1167	2.98×10^3	T	2.98×10^3	1.6	0.0
RX897	3.46×10^3	0.14×10^3	3.60×10^3	2.4	3.2
HS9977	1.30×10^3	0.38×10^3	1.68×10^3	NT	NT
3223	4.10×10^3	0.12×10^3	4.22×10^3	0.0	0.0
HY9899	2.74×10^3	0.14×10^3	2.88×10^3	NT	NT
32K61	5.49×10^3	0.22×10^3	5.71×10^3	3.8	0.0
8328	2.61×10^3	0.14×10^3	2.75×10^3	NT	NT
999	2.25×10^3	0.16×10^3	2.21×10^3	NT	NT
3085	4.96×10^3	0.28×10^3	5.24×10^3	NT	NT
TR1185	2.79×10^3	0.10×10^3	2.89×10^3	4.0	2.9
N7639 Bt	1.87×10^3	0.09×10^3	1.96×10^3	NT	NT
TVX21370	2.26×10^3	0.11×10^3	2.36×10^3	NT	NT
ATX770	4.81×10^3	T	4.81×10^3	1.2	11.1
DK687	2.89×10^3	0.04×10^3	3.00×10^3	T	13.2
TR702	4.30×10^3	T	4.30×10^3	NT	NT
3394	2.48×10^3	0.06×10^3	2.55×10^3	16.6	0.0
HY9919V	3.21×10^3	0.08×10^3	3.29×10^3	NT	NT
TR1154	5.68×10^3	0.32×10^3	6.00×10^3	2.4	4.2
TR1226	7.21×10^3	0.13×10^3	7.34×10^3	NT	NT
HS9843	7.74×10^3	0.18×10^3	7.92×10^3	3.0	0.0
AP9707	5.12×10^3	0.19×10^3	5.31×10^3	6.8	12.1
3163	2.76×10^3	0.08×10^3	2.84×10^3	10.8	1.4
DK706	5.17×10^3	0.07×10^3	5.25×10^3	NT	NT
TR1157	1.36×10^3	0.0	1.36×10^3	NT	NT
RX938	2.24×10^3	0.13×10^3	2.68×10^3	0.0	1.3
N7590Bt	3.83×10^3	0.09×10^3	3.92×10^3	NT	NT
3245	5.23×10^3	0.19×10^3	5.41×10^3	30.4	10.2
7770	2.27×10^3	0.07×10^3	2.34×10^3	NT	NT
AP9909	4.75×10^3	0.18×10^3	5.53×10^3	5.6	2.2
DK626	4.57×10^3	0.10×10^3	4.67×10^3	6.0	0.4
HS9944	10.4×10^3	0.11×10^3	10.5×10^3	NT	NT
RX813	3.22×10^3	0.20×10^3	3.42×10^3	NT	NT
TMF 113	9.11×10^3	0.48×10^3	9.59×10^3	NT	NT
3260	5.88×10^3	0.14×10^3	6.02×10^3	NT	NT
TR1066	6.99×10^3	0.08×10^3	7.06×10^3	NT	NT
8011	7.58×10^3	0.20×10^3	7.79×10^3	NT	NT
ATX721	3.91×10^3	0.19×10^3	4.01×10^3	NT	NT
TV2100	2.78×10^3	0.18×10^3	2.96×10^3	11.0	29.4
av $\pm$ SE	$(4.20 \pm 0.32) \times 10^3$	$(0.13 \pm 0.02) \times 10^3$	$(4.34 \pm 0.32) \times 10^3$	6.2 ± 1.6	4.5 ± 1.5
			Aflatoxin-Resistant Germplasm Lines		
Mp 420 $\times$ Tx601	537	72	609	0.0	0.0
Mp 715 $\times$ Tx601	NT	NT	NT	1.6	4.7
Mp 420 $\times$ Mp 80:04	NT	NT	NT	T	0.9
Mp 80:04 $\times$ GTMAS:gk	NT	NT	NT	2.4	1.2
Mp 420 $\times$ GTMAS:gk	NT	NT	NT	T	0.7
C12 $\times$ GT MAS:gk	NT	NT	NT	5.2	3.6
M182 $\times$ GT MAS:gk	NT	NT	NT	7.0	3.8
T115 $\times$ GT MAS:gk	NT	NT	NT	0.8	0.5
Mp 313E $\times$ Mp 80:04	NT	NT	NT	0.0	0.0
Mp313E $\times$ Mp420	279	132	411	1.8	4.0
Mp313E $\times$ Mp715	NT	NT	NT	0.0	0.0
Mp313E $\times$ Mp494	62	78	140	T	0.0
Mo18W $\times$ Mp313E	187	12	199	NT	NT
Ab24E $\times$ SC229	466	25	491	NT	NT
av $\pm$ SE	306 ± 88	64 ± 21	370 ± 88	1.6 ± 0.7	1.6 ± 0.5

[a] T = trace (below limit of detection), limit of detection = 0.5 μg/kg; NT = not tested; SE = standard error of the mean. [b] Levels of aflatoxins were determined by HPLC. Aflatoxin G_1 was not found except in RX938, 13.1 μg/kg. Aflatoxin G_2 was not found except in DK687, 7.4 μg/kg, and RX938, 18.9 μg/kg.

columns are cleaned for reuse as stated above. Each Neogen aflatoxin affinity column can be used to clean five samples in this manner. The 2 mL samples were evaporated to dryness in a Zymark evaporator as above.

HPLC Analysis of Aflatoxins. Using a modification of the method of DeVries and Chang (*30*), samples from the above cleanup procedure were treated with 50 μL of trifluoroacetic acid (TFA) and mixed on a vortex mixer for 20 s. The samples were then mixed with 3.95 mL of

acetonitrile/water/glacial acetic acid (10:9:1) (injection solvent) and placed in a Waters autosampler vial. A combined standards mixture with each of aflatoxins B_1, B_2, G_1, and G_2 was prepared by mixing Sigma standards so that a 100 μL aliquot in methanol contained sufficient quantities to inject from 0.5 to 2250 pg of each of the four aflatoxins. The standards were mixed in a 16 × 100 mm test tube, dried under nitrogen, mixed with 20 μL of TFA, diluted with 3.98 mL of injection solvent, and added to an autosampler vial. Samples and standards were programmed to inject and run for 20 min each at 2.5 mL/min through a Supelco 250 × 4.6 mm i.d. Discovery C_{18} column protected by a 20 × 4.0 mm, C_{18} i.d. precolumn, with both columns containing 5 μm beads. Sample data were collected using PC-Chrom software (H & A Scientific, Greenville, NC). Injections were made by a Waters 717 plus autosampler, and detection was effected by a Waters 474 scanning fluorescence detector set at 0.3 for sensitivity, 365 nm for excitation, and 440 nm for emission and using 12 mm slit width values. The mobile phase used to obtain separation was isocratic acetonitrile/water/acetic acid (24:76:1) delivered by a Waters 510 pump. Using this system we obtained a detection limit of 0.5 μg/kg for each of the four standard aflatoxins based on a signal-to-noise ratio ≥3 to 1. A trace was defined as a readily discernable peak (signal-to-noise ratio ≥ 1.5) that is below the limit of detection.

Fumonisin Cleanup. Cleanup of fumonisin samples utilized racks of up to 24 Bond-Elute SAX single-use columns (ChromTech, Inc., Apple Valley, MN) on a Supelco 24TM DL solid phase extraction tank system. The columns were prewashed with 5 mL of 100% methanol followed by 5 mL of methanol/water (75:25). Stored fumonisin samples were reconstituted in 10 mL of 70% methanol and passed through individual columns under vacuum. Extraneous materials in the samples were washed from the columns with 8 mL of 75% methanol followed by 3 mL of 100% methanol. Fumonisins were eluted from the columns with 14 mL of 0.5% acetic acid in methanol, dried in a Zymark evaporator, and stored for HPLC assay.

HPLC Analysis of Fumonisins. Samples were redissolved in 200 μL of 100% methanol, and a 30 μL aliquot was added to a 1 mL Waters autosampler vial, sealed, and placed in sequence in a Waters 96 vial autosampler rack. *o*-Phthaldialdehyde reagent for derivatizing fumonisins was prepared by dissolving 200 mg of *o*-phthaldialdehyde in 5 mL of 100% methanol, diluting into 25 mL of distilled water containing 0.95 g of disodium tetraborate, and mixing with 250 μL of β-mercaptoethanol. A 1 mL portion of this reagent was pipetted into selected autosampler vials placed in numbered positions as sources for a transfer program by the Waters 717 plus autosampler. Samples were derivatized by transferring 120 μL of *o*-phthaldialdehyde reagent from a source vial to a designated sample vial. After a 10 min programmed wait, the samples were injected onto a fumonisin-dedicated column of the same type as used for aflatoxin (see above). The wait period is critical, because derivative formation takes 10 min, but derivatives degrade rapidly after that time. Standards of fumonisin B_1 and B_2 (0.5−10 ng each) were treated in the same manner, giving a limit of detection of 0.5 mg/kg representing a signal-to-noise ratio ≥3 to 1. A trace was defined as a readily discernable peak (signal-to-noise ratio ≥ 1.5) that is below the limit of detection. The same software as for aflatoxins was used, programmed to receive samples at a 1 mL/min flow rate of methanol.NaH_2PO_4 (pH 3.33), 80:20, for 20 min. The same fluorescence detector as used for aflatoxins was used with the same sensitivity and slit width, but the excitation wavelength was set at 345 nm and the emission wavelength at 440 nm. The cleanup and HPLC analysis of fumonisins were both modifications of the method of Thiel et al. (*31*).

Liquid Chromatography/Mass Spectrometric Analysis of Aflatoxins. The analytical method used in detecting various aflatoxin compounds was liquid chromatography/atmospheric pressure chemical ionization/mass spectrometry (LC/APCI/MS). Briefly, extraction of corn samples was done using AOAC method 979.18 (*32*) with some modification. Ground corn samples (10 g) were weighed into a 50 mL capped centrifuge tube and 30 mL of MeOH/H_2O (4:1, v/v) was added. The mixture was shaken for 1 h, and then 15 mL of the supernatant was mixed with 15 mL of salt solution [60 g of NaCl, 60 g of Zn-$(CH_3COO)_2$, 1.5 mL of CH_3COOH in 400 mL of H_2O] in a 50 mL centrifuge tube and shaken for 10 s. A 15 mL aliquot of the mixture was transferred into a 20 mL vial, and 3 mL of benzene was added and shaken for 10 s. An aliquot (2 mL) of the benzene layer was placed into a 4 mL vial and evaporated to dryness under nitrogen. The LC/APCI/MS analysis was done in selected ion monitoring (SIM) mode on a Finnigan Mat LCQ system (ThermoQuest, San Jose, CA). The HPLC column was 5 cm × 4.6 mm i.d. packed with 5 μm reversed phase C_{18} (Supelco, Bellefonte, PA). The mobile phase contained 1% AcOH plus 8−72% MeOH in H_2O. Gradient elution of aflatoxins was done by keeping MeOH at 8% for 1 min, increasing to 40% in 19 min, holding at 40% for 5 min, and then increasing to 72% in 5 min. Ions at *m*/*z* 313, 315, 329, and 331 were monitored for protonated molecular ions of aflatoxins B_1, B_2, G_1, and G_2, respectively. The percentages of each aflatoxin type were calculated as the area under the curve for each type as a percentage of the total area under the curve for all aflatoxin types detected in the sample.

Liquid Chromatography/Mass Spectrometric Analysis of Fumonisins. The analytical method used in detecting various fumonisin derivatives was liquid chromatography/electron spray ionization/mass spectrometry (LCI/ESI/MS) (*33*). Briefly, ground corn samples (10 g) were extracted with 30 mL of MeOH/H_2O (3:1, v/v) in a capped 50-mL centrifuge tube shaker for 1 h. Sample cleanup was done by using the method of Thiel et al. (*31*). LC/ESI/MS analysis was done on the Finnigan Mat LCQ system in full-scan mode. The same HPLC column used for aflatoxin analysis was used for fumonisin analysis. The mobile phase contained 1% AcOH plus 8−64% MeOH in H_2O. Gradient elution of fumonisins was done by holding the MeOH concentration at 8% for 1 min, followed by increasing MeOH concentration to 64% in 19 min, and holding the MeOH concentration at 64% for 10 min. Ions at *m*/*z* 722, 706, 690, 690, and 676 were monitored for molecular ions of fumonisins B_1, B_2, B_3, B_4, and C_4, respectively. The percentages of each fumonisin type were calculated as the area under the curve for each type as a percentage of the total area under the curve for all fumonisin types detected in the sample.

ELISA Analysis of Aflatoxins and Fumonisins. To facilitate comparison of the results obtained in the present study with results obtained by the widely used commercial ELISA assays, selected corn samples from 1998 were extracted and subjected to cleanup as described for HPLC analysis. Aliquots were analyzed for the presence of total aflatoxins and total fumonisins with commercially available quantitative ELISA assay kits (Neogen Inc., Lansing, MI) used according to the manufacturer's instructions (*34*). Both groups of toxins were confirmed qualitatively by TLC, using chloroform/acetone (97:3) as the developing solvent for aflatoxins (*35*) and ethyl acetate/acetic acid/water (60:30:1) as the developing solvent for fumonisins B_1 and B_2. For detection of aflatoxins, TLC plates (20 × 20 cm precoated with 0.25 mm silica gel with gypsum binder, Alltech Associates, Deerfield, IL) were viewed under long-wavelength UV (365 nm) illumination. For detection of fumonisins TLC plates were sprayed with *p*-anisaldehyde as described in detail by Gelderblom et al. (*10*).

Deoxynivalenol and Zearalenone Analysis. Corn samples from 1998 were extracted and subjected to cleanup as described for HPLC analysis. Aliquots were analyzed for the presence of deoxynivalenol and zearalenone with commercially available quantitative ELISA assay kits (Neogen Inc.) used according to the manufacturer's instructions. Deoxynivalenol and zearalenone were confirmed by TLC and gas chromatography/mass spectroscopy methods as described in detail by Mirocha et al. (*36*).

Statistical Analysis. The correlation of aflatoxin levels with fumonisin levels was investigated by linear regression analysis, and significance was evaluated by analysis of variance using Statview statistical software. Comparisons of mycotoxin levels observed under various conditions was evaluated for significance by Student's unpaired *t*-test using the statistical package included in Microsoft Excel 97 software.

RESULTS AND DISCUSSION

Both aflatoxin and fumonisin were present at very high levels in all corn samples harvested from the 45 commercial hybrids grown in Starkville in 1998 (**Tables 1** and **2**). Total aflatoxins averaged $(4.34 \pm 0.32) \times 10^3$ mg/kg (mean ± standard error) and ranged from 0.95×10^3 to 10.5×10^3 μg/kg. Aflatoxin B_1

Table 2. Levels of Fumonisins and Deoxynivalenols in Corn Samples Collected in 1998 and 1999[a]

	1998, Starkville				1999	
	fumonisins (mg/kg)			deoxynivalenols	fumonisins (mg/kg)	
hybrid no.	B_1	B_2	total	(mg/kg)	Starkville	Stoneville
			Commercial Hybrids			
1177	2.2	T	2.2	0.0	NT	NT
5688	5.0	0	5.0	0.0	2.3	3.0
5510A	10.9	1.2	12.1	0.2	0.5	0.8
8460	18.0	0	18.0	0.0	2.0	2.5
TV2930	8.7	T	8.7	0.0	NT	NT
DK683	T	4.9	4.9	0.0	2.0	1.2
5670	16.7	1.2	17.9	0.1	3.3	4.8
TR1167	12.8	0.3	13.1	0.0	2.2	2.4
RX897	10.9	1.5	12.4	0.0	2.3	3.3
HS9977	0.6	0	0.6	0.0	NT	NT
3223	30.0	5	35.0	0.0	1.0	0.8
HY9899	T	0	T	0.2	NT	NT
32K61	T	2.1	2.1	0.3	2.2	3.1
8328	T	0	T	0.0	NT	NT
999	0.4	T	0.4	0.0	NT	NT
3085	13.0	0	13.3	0.0	NT	NT
TR1185	8.4	0	8.4	0.0	3.0	1.4
N7639 Bt	9.9	1.9	11.8	0.0	NT	NT
TVX21370	0.9	0	0.9	1.3	NT	NT
ATX770	16.8	T	16.8	0.0	2.5	2.1
DK687	18.9	T	18.9	0.0	1.4	0.6
TR702	0.7	T	0.7	0.2	NT	NT
3394	16.1	1.5	17.6	1.5	2.6	3.6
HY9919V	8.6	1.3	9.9	0.3	NT	NT
TR1154	10.6	1.5	12.1	0.0	1.8	2.4
TR1226	15.8	4.3	20.1	0.0	NT	NT
HS9843	14.1	4.0	18.1	0.3	1.9	1.1
AP9707	2.0	T	2.2	0.2	4.0	2.7
3163	13.3	5.9	19.2	0.0	4.8	0.8
DK706	5.9	T	5.9	0.0	NT	NT
TR1157	17.7	8.6	26.3	0.8	NT	NT
RX938	4.5	T	4.5	0.0	0.9	1.7
N7590Bt	11.4	T	11.4	0.0	NT	NT
3245	17.5	2.0	19.5	0.0	4.5	4.2
7770	14.1	0	14.1	0.0	NT	NT
AP9909	13.0	3.0	16.0	0.0	2.3	2.9
DK626	T	10.7	10.7	0.0	3.3	4.0
HS9944	9.1	T	9.1	0.0	NT	NT
RX813	14.0	1.6	15.6	0.0	NT	NT
TMF 113	0.9	0	0.9	0.0	NT	NT
3260	10.9	8.3	19.2	0.2	NT	NT
TR1066	4.2	T	4.2	0.1	NT	NT
8011	14.6	2.4	17.0	0.0	NT	NT
ATX721	15.4	4.2	19.6	0.1	NT	NT
TV2100	8.8	1.0	9.8	0.6	3.1	3.3
av ± SE	9.5 ± 1.0	1.7 ± 0.4	11.2 ± 1.2	0.14 ± 0.05	2.5 ± 0.2	2.4 ± 0.3
			Aflatoxin-Resistant Germplasm Lines			
Mp 420 × Tx601	6.5	0.0	6.5	0.1	0.5	0.4
Mp 715 × Tx601	NT	NT		NT	0.5	0.5
Mp 420 × Mp 80:04	NT	NT		NT	3.0	0.7
Mp 80:04 × GT MAS:gk	NT	NT		NT	2.0	1.1
Mp 420 × GT MAS:gk	NT	NT		NT	2.2	0.6
C12 × GT MAS:gk	NT	NT		NT	0.9	0.4
M182 × GT MAS:gk	NT	NT		NT	0.8	2.0
T115 × GT MAS:gk	NT	NT		NT	2.0	2.4
Mp 313E × Mp 80:04	NT	NT		NT	0.5	0.8
Mp313E × Mp420	2.0	0.0	2.0	0.0	0.6	0.8
Mp313E × Mp715	NT	NT		NT	0.8	0.6
Mp313E × Mp494	1.7	0.0	1.7	0.1	0.8	1.3
Mo18W × Mp313E	2.2	0.0	2.2	0.0	NT	NT
Ab24E × SC229	7.8	0.0	7.8	0.0	NT	NT
av ± SE	4.0 ± 1.3	0.0	4.0 ± 1.3	0.04 ± 0.02	1.2 ± 0.2	1.0 ± 0.2

[a] Levels of fumonisins were determined by HPLC in 1998 and by ELISA in 1999. T = trace (below limit of detection); NT = not tested; SE = standard error of the mean. Deoxynivalenol was determined by ELISA and confirmed by GC/MS. Zearalenone was not detected.

was present in 100% of samples, and aflatoxin B_2 was found in 88% (**Figure 1**). Total fumonisins averaged 11.2 ± 1.2 mg/kg and ranged from 0.4 to 35 mg/kg. Fumonisins B_1, B_2, and B_3 were present in all samples, and fumonisins B_4 and C_4 were also present in 95% of samples (**Figure 1**). Deoxynivalenol (**Figure 1**) was detected in 33% of these samples for an average of 0.14 ± 0.05 mg/kg with a range from 0 to 1.5 mg/kg. However, no zearalenone (**Figure 1**) was found (**Table 2**). Corn

Table 3. Relative Amounts of Fumonisin and Aflatoxin Subtypes Measured in Selected Corn Samples by LC/MS Methods

	fumonisin levels[a]						aflatoxin levels[a]		
hybrid no.	total (mg/kg)	% B_1	% B_2	% B_3	% B_4	% C_4	total (μg/kg)	% B_1	% B_2
				1998, Starkville, MS					
HS9843	19	47.9	15.2	5.58	30.8	0.42	8.40×10^3	97.5	2.5
N7639Bt	8	72.3	20.3	1.13	6.25	0.13	3.24×10^3	96.2	3.8
3394	15	77.7	12.0	7.40	2.53	0.27	2.56×10^3	95.8	4.2
AP 9909	15	65.1	20.7	8.33	5.93	0.0	5.60×10^3	96.4	3.6
3223	25	70.2	18.3	8.00	3.04	0.44	3.60×10^3	96.7	3.3
TR 1226	15	63.4	19.3	11.5	5.07	0.73	6.80×10^3	95.9	4.1
3245	19	64.4	18.0	12.3	4.68	0.68	6.40×10^3	96.6	3.4
8011	17	67.4	18.7	9.9	3.59	0.35	7.20×10^3	96.4	3.6
RX 813	16	65.6	21.2	8.9	3.87	0.50	4.80×10^3	95.8	4.2
				1999, Starkville, MS					
AP 9707	4.0	67.3	15.0	14.0	3.50	0.0	4	100	0.0
3245	3.0	65.3	16.3	13.3	4.33	0.67	23	100	0.0
				1999, Stoneville, MS					
AP 9707	1.3	66.9	20.0	10.0	3.08	0.0	27	100	0.0
3245	1.5	66.0	15.3	15.3	3.97	0.0	8	100	0.0

[a] Total fumonisins and aflatoxins in each corn sample were determined by ELISA methods. Percentages of individual fumonisins and aflatoxins were determined by LC-MS.

samples from all aflatoxin-resistant germplasm lines were contaminated with aflatoxins as well, but there was a highly significant ($p < 0.00001$; Student's unpaired *t* test), almost 12-fold reduction in total aflatoxins to an average of 370 ± 88 μg/kg ranging from 140 to 609 μg/kg (**Table 1**). Total fumonisin levels were also significantly ($p < 0.05$; Student's unpaired *t* test) reduced in corn harvested from aflatoxin-resistant germplasm lines, averaging 4.0 ± 1.3 mg/kg (1.7−7.8 mg/kg). However, the ~3-fold reduction in fumonisins in aflatoxin-resistant germplasm lines is much less dramatic than that for aflatoxins.

In 1999 aflatoxin contamination in corn harvested in Starkville, MS, was very significantly (Student's unpaired *t* test) reduced from the previous year, by 700-fold to an average of 6.2 ± 1.6 μg/kg (0−30.4 μg/kg) ($p < 0.0001$) in commercial hybrids and by 231-fold to an average of 1.6 ± 0.7 μg/kg (0−7 μg/kg) in aflatoxin-resistant germplasm lines ($p < 0.0001$). Similarly reduced aflatoxin levels were also observed for plantings treated in the same manner in Stoneville, MS, 210 km to the west, that is, reduction to averages of 4.5 ± 1.5 μg/kg (0−29.4 μg/kg) in commercial hybrids ($p < 0.0001$) and 1.6 ± 0.5 μg/kg (0−4.7 μg/kg) in aflatoxin-resistant germplasm lines ($p < 0.0001$) (**Table 1**). Total aflatoxin levels in 1999 were significantly lower in corn samples from aflatoxin-resistant germplasm lines than in samples from commercial hybrids in Starkville and Stoneville ($p < 0.01$; Student's unpaired *t* test). Total fumonisin levels in corn harvested in 1999 in Starkville averaged 2.5 ± 0.2 mg/kg (0.5−4.8 mg/kg) in commercial hybrids, which were significantly higher than in aflatoxin-resistant germplasm lines, which averaged 1.2 ± 0.2 mg/kg (0.5 ± 3.0 mg/kg) ($p < 0.0001$; Student's unpaired *t* test). In Stoneville fumonisin levels averaged 2.4 ± 0.3 mg/kg (0.6−4.8 mg/kg) in commercial hybrids, which was significantly higher than in aflatoxin-resistant germplasm lines, which averaged 1.0 ± 0.2 mg/kg (0.4−2.4 mg/kg) ($p < 0.0001$; Student's unpaired *t* test). Neither total aflatoxin levels nor total fumonisin levels in corn samples differed significantly ($p > 0.2$; Student's unpaired *t* test) between Starkville and Stoneville for either commercial hybrids, aflatoxin-resistant germlines, or all hybrids considered together.

Nine representative corn samples from each of the 1998 and 1999 growing seasons (two from each location) were analyzed for fumonisins by a widely used ELISA assay method, and the relative amounts of fumonisins B_1, B_2, B_3, B_4, and C_4 (**Figures 1** and **2**) were quantified by LC/ESI/MS (**Table 3**). These toxins were found in easily detectable levels in corn samples from 1998. However, when corn samples from 1999 were analyzed for fumonisins B_1, B_2, B_3, B_4, and C_4 using the same methods,

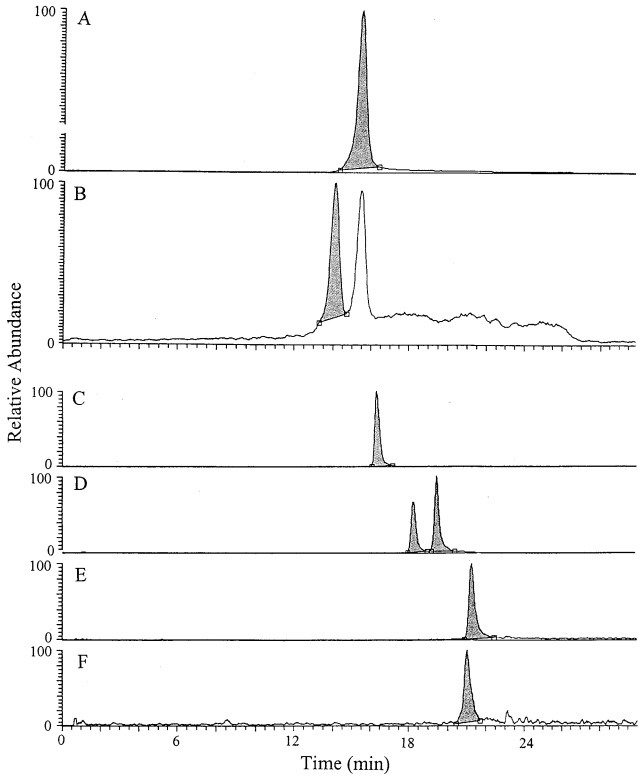

Figure 2. LC/APCI/MS chromatograms at *m/z* 313 for aflatoxin B_1 (A, retention time = 15.46 min) and at *m/z* 315 for aflatoxin B_2 (B, shaded, retention time = 14.11 min) present in a crude extract from a corn hybrid (hybrid no. 3245) naturally infected with *Fusarium* species and inoculated with *A. flavus* at a rate of 9×10^7 spores/mL weekly for 5 weeks. Panels C–F show LC/ESI/MS chromatograms at *m/z* 722 for fumonisin B_1 (C, retention time = 16.34 min), at *m/z* 706 for fumonisin B_2 (D, right, retention time = 19.45 min) and fumonisin B_3 (D, left, retention time = 18.25 min), at *m/z* 690 for fumonisin B_4 (E, retention time = 21.27 min), and at *m/z* 676 for fumonisin C_4 (F, retention time = 21.03 min) in the same extract. The derivatives eluted at the indicated retention times (minutes).

Table 4. Summary of Temperature and Precipitation Observed at the National Weather Service Cooperative Observed Network Weather Site in Starkville, MS, during the 1998 and 1999 Growing Seasons (May–July)

year	T_{max}, av max air temp (°C)	T_{min}, av min air temp (°C)	no. of days T_{min} > 20 °C	consecutive days T_{min} > 20 °C	total DD20[a]	cumulative rain (cm)
1998	32.8	20	57	41	+589	24.6
1999	30.6	18.9	41	20	+488	27.2

[a] DD20 = growing degree units = $(T_{max} + T_{min})/2 - 20$ °C. Total DD20 is the sum of the DD20 values over the growing season.

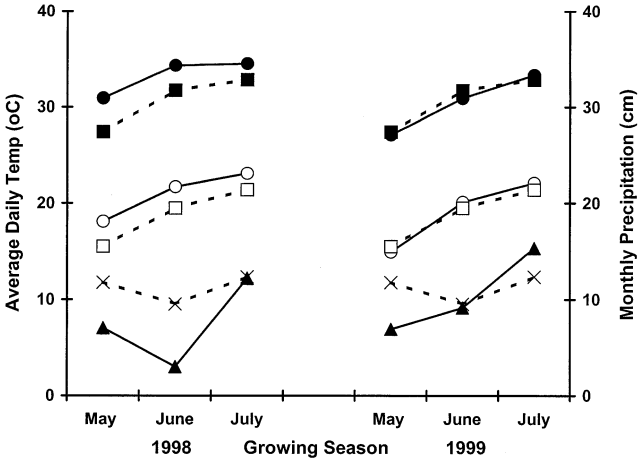

Figure 3. Average daily maximum (solid symbols) and minimum (open symbols) temperatures in Starkville, MS, during the corn growing seasons of 1998 and 1999 (circles) and the average since 1930 (squares). The monthly precipitation is given for the corn growing seasons of 1998 and 1999 (diamond) and since 1930 (×).

some derivatives were detected only at very low levels and other derivatives were not present (**Table 3**). The same 13 samples were analyzed for aflatoxins by a widely used ELISA assay method, and the relative amounts of aflatoxin B_1 and B_2 (**Figures 1** and **2**) were quantified by LC/APCI/MS. Both aflatoxins B_1 and B_2 were found in all corn samples from 1998 at high levels exceeding 20 μg/kg, but in corn samples from the 1999 test, the levels of aflatoxins B_1 and B_2 were 0−27 μg/kg, respectively (**Table 3**).

The high levels of grain contamination by both aflatoxin and fumonisin in 1998 in Mississippi have been attributed to the effects of heat and drought stress on the corn plants (*9*). Most of the southeastern United States experienced drought conditions from May 1998 through October 2000. One of the most intense periods of heat and drought stress for the Lower Mississippi River Valley occurred in 1998 during three months of the corn growing season (May, June, and July). Corn is typically planted in the last week of March, and it reaches physiological maturity (blacklayer) in mid-July to mid-August. Most of the vegetative and reproductive growth occurs in the May−July time frame. In Starkville, MS, the 1998 growing season was characterized by well above normal maximum and minimum temperatures and below normal precipitation (**Figure 3**). May, June, and July, 1998, were, respectively the fourth, fifth, and seventh warmest on record, and the combined growing period was the warmest on record (since 1930).

It was felt that these unusual weather conditions provided a unique opportunity to investigate the effects of environmental stress conditions on mycotoxin production in corn in the field by repeating the 1998 study of aflatoxin and fumonisin production in subsequent years until more nearly normal heat and drought stress conditions occurred. The 1999 growing season proved to be such a year. In May−July, 1999, Starkville experienced much closer to normal temperature and precipitation (**Figure 3**). In Stoneville, where additional plantings were conducted in 1999, weather conditions were similar. Thus, heat and drought stress conditions for corn production were moderate in 1999.

Previous research has shown that growth of aflatoxin- and fumonisin-producing microorganisms is retarded or stagnant at temperatures below 20 °C (*6*, *7*). In Starkville the 1998 growing season was characterized by both higher maximum (2.2 °C) and higher minimum (1.1 °C) air temperatures than in 1999 (**Table 4**). Of particular concern are minimum air temperatures >20 °C, which are suspected to be more important in permitting fungal growth, because they occur at night when the plant is in negative energy balance and less able to defend itself. As summarized in **Table 4**, in the 1998 growing season the minimum air temperature was >20 °C for substantially more days and longer consecutive periods than in 1999. Growing degree units using 20 °C as the base temperature [DD20 = (maximum temperature + minimum temperature)/2 − 20 °C] were calculated and summed over the May−July time period to quantify variances in minimum air temperature in 1998 and 1999. In the 1998 growing season the totaled DD20 values were +589 degree units, 101 more than in 1999. These temperature indices clearly indicate that greater heat stress occurred in 1998. Cumulative rainfall differed by only 2.6 cm, although 1998 was characterized by fewer but larger rain events with longer intervals between rains, whereas in 1999 rain events were smaller but more frequent and much more uniformly spaced. Larger rain events result in more runoff, so that less total water is captured by the soil for use by the crop. However, the effects of drought stress were minimized with supplemental irrigation in both years.

Plant stress due to weather conditions would seem to be a very significant factor for both aflatoxin and fumonisin production in Mississippi. The hot, dry conditions present in Mississippi in 1998 proved to be ideal for the production of large amounts of both fumonisins and aflatoxins. These results suggest that it is important to monitor corn for contamination with both toxins during hot, dry conditions. These results also suggest that it may be possible to simultaneously control both of these toxins with similar cultural practices that reduce plant stress. Given that drought stress was relatively mild in 1998, and not greatly different in 1999, the results are consistent with heat stress alone (i.e., in the absence of substantial drought stress) being sufficient to permit high aflatoxin and fumonisin production. Because both the maximum (day) temperature and the minimum (night) temperature were markedly elevated in 1998, it is not possible to draw conclusions about the relative importance of day versus night temperature. Further research under greenhouse conditions may be needed to more completely define the types of stress that affect mycotoxin production in corn.

Corn is a major staple food crop in the United States as well as many countries throughout the world. The livestock industry also depends heavily on corn as feed. Due to the risks of fumonisins to human and animal health (*11*−*13*, *17*, *18*), the

FDA has recently proposed a guideline of a maximum of 2 mg/kg in corn and corn products for human consumption (*20*). Aflatoxin has long been monitored by the FDA, and a level of 20 μg/kg has been set as the limit for corn contamination with aflatoxin. In 1998 all inoculated corn hybrids listed in **Table 1** exceeded the FDA-recommended levels for aflatoxin, and 84.4% of these hybrids exceeded the FDA-recommended levels for fumonisins (**Table 2**). In 1999, the levels of both groups of toxins were much lower in comparison to 1998. In 1999, aflatoxin levels were 0−30.4 μg/kg with only two samples exceeding FDA-recommended levels. Similarly, fumonisin levels were 0.5−4.8 mg/kg, and only 13 samples exceeded FDA-recommended levels.

This study confirms previous observations (*38*; W. Mubatanhema, Z. Jurjevic, D. M. Wilson, B. Evans, N. Widstrom, and F. I. Meredith, unpublished results) that high levels of aflatoxin can coexist with fumonisins in corn. It has been suggested that there is a negative relationship between *Aspergillus* spp. and *Fusarium* spp. in corn (*38*). However, in the present study there was no significant positive or negative correlation (linear regression analysis/analysis of variance) between measured aflatoxin B_1 levels and fumonisin B_1 levels in Starkville in 1998 ($r^2 = 0.037$; $0.1 < p < 0.25$) or in Starkeville and Stoneville in 1999 ($r^2 = 0.01$; $p > 0.25$). Thus, the natural infection with *Fusarium* spp. did not appear to protect against the production of aflatoxin by *A. flavus* at an inoculum size used in this study. Differences between these observations and those of Marin et al. (*38*) may result from either lower levels of *Fusarium* spp. infestation in the present study or high levels of *A. flavus* infestation associated with intentional inoculation.

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