Aspergillus flavus Infection and Aflatoxin Accumulation in Resistant and Susceptible Maize Hybrids

G. L. Windham and W. P. Williams, USDA-ARS, Crop Science Research Laboratory, P.O. Box 5367, Mississippi State, MS 39762

ABSTRACT

Windham, G. L., and Williams, W. P. 1998. *Aspergillus flavus* infection and aflatoxin accumulation in resistant and susceptible maize hybrids. Plant Dis. 82:281-284.

Field studies were conducted for 2 years in Mississippi to monitor maize kernel infection and aflatoxin accumulation caused by *Aspergillus flavus* at various times during the growing season. Hybrids resistant and susceptible to *A. flavus* were compared to determine temporal differences in infection and aflatoxin levels. The resistant hybrids tested were Mo18W $\times$ Mp313E, Mp420 $\times$ Tx601, and SC54 $\times$ SC76; and the susceptible hybrids tested were GA209 $\times$ Mp339, Mp307 $\times$ Mp428, and Mp68:616 $\times$ SC212M. The top ear of each plant was inoculated with a suspension containing *A. flavus* conidia at 7 days after mid silk (50% of the plants in a plot had silks emerged) using the side needle technique. Inoculated ears were harvested 35, 42, 49, 56, and 63 days after mid silk to determine kernel infection by *A. flavus* and aflatoxin contamination. Differences in aflatoxin levels between resistant and susceptible hybrids occurred in all harvest dates. However, significant differences between resistant and susceptible hybrids for kernel infection were not observed until 42 days after mid silk. Differences between resistant and susceptible hybrids occurred for kernel infection and aflatoxin concentrations 49, 56, and 63 days after mid silk. Incidence of kernel infection (8.1% for GA209 $\times$ Mp339) was the highest 49 days after mid silk, and aflatoxin concentrations (510 ng/g for Mp307 $\times$ Mp428) were the highest 63 days after mid silk. Maximum differences between resistant and susceptible hybrids for aflatoxin levels were observed 63 days after mid silk. Two of the resistant hybrids, Mo18W $\times$ Mp313E and Mp420 $\times$ Tx601, had significantly less aflatoxin than the three susceptible hybrids 63 days after mid silk.

Additional keywords: corn, mycotoxin, resistance, *Zea mays*

Infection of maize kernels by *Aspergillus flavus* and subsequent accumulation of aflatoxin is a chronic economic and health safety problem in the southeastern United States and can be a problem in the Corn Belt (2-4,7-9,17,21). Aflatoxins are some of the most potent carcinogens produced in nature. Aflatoxin B₁ is the most commonly found aflatoxin in maize and is also the most potent. This aflatoxin has been the subject of numerous toxicological studies (3). The United States Food and Drug Administration has a tolerance of 20 ng/g for aflatoxin B₁ in maize (3). Grain that exceeds this level cannot be shipped through

interstate commerce. Many countries have a tolerance of 10 ng/g in food supplies, while several countries have a zero tolerance for aflatoxin contamination (3).

Much information on *A. flavus* infection of maize and subsequent aflatoxin contamination has been published over the last 20 years. Several factors associated with increased aflatoxin formation in maize are drought stress (2,11), high temperatures (11), and insects (9,10). Studies have been conducted to determine the time and location of *A. flavus* infection in maize (7,12,14,18-20). Payne et al. (12) followed the rate of aflatoxin accumulation in ears of a susceptible maize hybrid. Aflatoxin appeared 1 week after inoculation and generally peaked 7 to 9 weeks after inoculation. No information on the temporal changes in *A. flavus* infection and the initiation of aflatoxin production in resistant maize compared with susceptible maize is available. Information on the timing of kernel infection and aflatoxin accumulation is needed to help researchers determine when to harvest maize genotypes to accurately determine levels of resistance. Field studies were conducted 2 years to monitor kernel infection and aflatoxin accumulation

in hybrids resistant and susceptible to *A. flavus* to determine when maximum differences occurred.

MATERIALS AND METHODS

Three resistant hybrids (Mo18W $\times$ Mp313E, Mp420 $\times$ Tx601, and SC54 $\times$ SC76) and three susceptible hybrids (GA209 $\times$ Mp339, Mp307 $\times$ Mp428, and Mp68:616 $\times$ SC212M) were used in this study based on performance in other studies (1,14,22). These hybrids were grown at the Plant Science Farm, Mississippi State, Mississippi, in a randomized complete block design with six replications in 1994 and 1995. Planting dates were 14 April 1994 and 7 April 1995. Single-row, 5.1-m plots spaced 0.96 m apart were thinned to 20 plants per plot. Plots received supplemental irrigation during each growing season to limit drought stress.

A. flavus isolate NRRL 3357, which is known to produce aflatoxin in maize grain (13), was used as inoculum both years. Inoculum was increased on sterile maize cob grits in 500-ml flasks, each containing 50 g of grits and 100 ml of H₂O, and incubated at 28°C. Conidia were washed from the grits using sterile distilled water containing 20 drops of Tween 20 per liter and filtered through four layers of sterile cheesecloth. The concentration of conidia was determined with a hemacytometer and adjusted with sterile distilled water to 9×10^7 conidia per ml. Inoculum not immediately used was refrigerated at 4°C. Hybrids were inoculated 7 days after mid silk (50% of the plants in the plots had silks emerged) using the side needle technique (22). The top ear of each plant was inoculated with a 3.4-ml suspension containing 3×10^8 *A. flavus* conidia.

Inoculated ears were harvested 35, 42, 49, 56, and 63 days after mid silk. Grain moisture was measured using a Dickey-John Grain Moisture Tester (Auburn, IL). Ears were then dried at 38°C for 7 days and machine shelled. Grain samples from each row were poured into a sample splitter twice to mix the grain. Half of the grain was used for aflatoxin analyses, and the other half was used for determination of kernel infection. Grain samples used in aflatoxin analyses were ground using a Romer mill (Union, MO). Aflatoxin contamination in 50-g subsamples from each

Corresponding author: G. L. Windham
E-mail: gwindham@dorman.msstate.edu

Contribution of the USDA-ARS in cooperation with the Mississippi Agricultural and Forestry Experiment Station, Mississippi State 39762.

Accepted for publication 3 December 1997.

Publication no. D-1998-0109-01R

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1998.

plot was determined using the Vicam Aflatest (Watertown, MA). This procedure can detect aflatoxins (B₁, B₂, G₁, G₂) at concentrations as low as 2 ng/g.

Kernels evaluated for *A. flavus* infection were surface sterilized by dipping momentarily (5 s) in 70% ethanol, soaking for 3 min in 1.5% NaOCl, and rinsing in sterile distilled water. The kernels were placed in 100-mm petri dishes (13 kernels per dish, 390 kernels per plot) on Czapek solution agar amended with 7.5% NaCl to restrict growth of other fungi and bacteria. After 7 days at 28°C, kernels were examined for *A. flavus* growth.

Data were subjected to analysis of variance. Since there was no genotype by year interaction for kernel infection or aflatoxin accumulation, data from both years were combined for further analyses and for presentation. Means were compared by the least significant difference test (LSD) at $P = 0.05$. Means of resistant and susceptible hybrids for kernel infection and aflatoxin accumulation were compared using orthogonal contrasts.

RESULTS

Differences ($P = 0.05$) in *A. flavus* kernel infection were not found until the second harvest date (42 days after mid silk) (Table 1). Two of the resistant hybrids, Mo18W × Mp313E and Mp420 × Tx601, had significantly lower incidence of kernel infection than did Ga209 × Mp339 and Mp307 × Mp428. At 49 days after mid silk, all of the resistant hybrids had significantly ($P = 0.05$) less kernel infection than the susceptible hybrids. Mo18W × Mp313E and Mp420 × Tx601 had the lowest levels of kernel infection at the last two harvest dates. Incidence of kernel infection for two susceptible hybrids (GA209 × Mp339 and Mp307 × Mp428) peaked 49 days after mid silk. Kernel infection in the other susceptible hybrid (Mp68:616 × SC212M) was the greatest 56 days after mid silk.

When the means of resistant and susceptible hybrids were compared using orthogonal contrasts, no differences were found for kernel infection on the first harvest date (Fig. 1A). However, significant differences ($P = 0.05$) between resistant and susceptible hybrids were observed at the last four harvest dates. Maximum differences in kernel infection between resistant and susceptible hybrids were found 49 days after mid silk (Fig. 1A).

Aflatoxin levels differed significantly ($P = 0.05$) between resistant and susceptible hybrids at the first harvest date (35 days after mid silk) (Table 2). All of the resistant hybrids had lower aflatoxin concentrations than two of the susceptible hybrids (GA209 × Mp339 and Mp307 × Mp428). Aflatoxin concentrations in Mp68:616 × SC212M at the first harvest were comparable to those in the three resistant hybrids. Generally, for the other harvest dates, aflatoxin accumulated at a much slower

Table 1. *Aspergillus flavus* kernel infection (%) of resistant and susceptible maize hybrids at five harvest dates

Hybrid	Days after mid silk				
	35	42	49	56	63
Resistant					
Mo18W × Mp313E	3.8 ^a	3.2	2.9	2.9	3.2
Mp420 × Tx601	3.9	2.1	3.2	2.2	2.7
SC54 × SC76	5.0	4.3	3.7	5.7	6.6
Susceptible					
GA209 × Mp339	3.5	6.4	8.1	7.0	5.7
Mp307 × Mp428	5.4	6.3	7.3	6.1	5.8
Mp68:616 × SC212M	3.0	4.8	7.7	8.0	7.5
LSD ($P = 0.05$)	NS ^b	2.8	2.7	2.0	2.1

^a Data are mean values of 12 replicates.

^b No significant difference among hybrids.

Fig. 1. Kernel infection (A) and aflatoxin accumulation (B) in resistant and susceptible maize hybrids at five harvest dates. Each bar represents the mean of three genotypes. An asterisk indicates a significant difference ($P = 0.05$) in the orthogonal contrast: resistant vs. susceptible.

rate in the resistant hybrids than in the susceptible hybrids. By the last harvest date, the resistant hybrid (Mo18W × Mp313E) had the lowest concentration of aflatoxin (93 ng/g), and the susceptible hybrid (Mp307 × Mp428) had the highest (510 ng/g). For most harvest dates, the resistant hybrids Mo18W × Mp313E and Mp420 × Tx601 had significantly lower aflatoxin concentrations than did the susceptible GA209 × Mp339 and Mp307 × Mp428 hybrids.

When data for the three resistant hybrids were combined and data for the three susceptible hybrids were combined, differences ($P = 0.05$) in aflatoxin concentrations between the two groups of hybrids were noted at all harvest dates (Fig. 1B). Aflatoxin accumulation was always higher in the susceptible group of hybrids. Maximum differences in aflatoxin concentrations were observed at the last harvest date (63 days after mid silk).

Kernel infection and aflatoxin concentrations were each negatively correlated ($P = 0.0001$) with grain moisture. Over time, as the maize dried down, kernel infection and aflatoxin levels increased. Moisture levels in the resistant hybrids were higher than moisture levels in susceptible hybrids at the early harvest dates (Fig. 2). As the season progressed, moisture levels in the two groups began to converge, reaching the

same levels of moisture 63 days after mid silk.

DISCUSSION

Aflatoxin accumulation in susceptible maize genotypes has been monitored in previous studies (7,12,14,18,20). Thompson et al. (18) found aflatoxin in maize kernels 2 days postinoculation, and aflatoxin concentrations peaked 9 days postinoculation. In another study (7), aflatoxin concentrations peaked 30 days after inoculation. Others have found that aflatoxin levels increased linearly throughout the growing season and peaked near physiological maturity (12, 20). Our findings agree more with the latter studies with a linear increase in aflatoxin accumulation. In our study, aflatoxin levels for all resistant and susceptible hybrids peaked at the last harvest. Payne et al. (12) reported a significant drop in aflatoxin levels close to their last harvest dates. This drop in aflatoxin concentration did not occur in our study.

Two of the hybrids did not exhibit the same level of resistance or susceptibility to *A. flavus* as reported in other studies. The resistant hybrid SC54 × SC76 had low levels of *A. flavus* kernel infection when inoculated with a pinbar, which is a severe inoculation technique (22). In our study, however, the incidence of kernel infection in SC54 × SC76 63 days after mid silk was comparable to that of the three susceptible hybrids. Also, the susceptible hybrid Mp68:616 × SC212M had less kernel infection in our study than that reported by Zummo and Scott (22). After inoculation with *A. flavus* using the side needle technique, kernel infection of Mp68:616 × SC212M in that study was over 20%. In our study, at the initial harvest date, this hybrid had numerically the lowest level of infection of any of the hybrids; however, after the second harvest date, the levels were indicative of the expected susceptibility. The resistant hybrid Mp420 × Tx601 demonstrated high levels of resistance in our study and also in other studies (1,14,22).

Few characteristics that cause certain hybrids to be resistant to *A. flavus* kernel infection and limit subsequent aflatoxin

contamination have been documented (5,6). Kernel moisture of the hybrids was monitored to determine if moisture levels played a role in resistance. Different rates of drying by resistant and susceptible hybrids apparently contributed little to the differences in kernel infection and aflatoxin levels we observed. At the first harvest dates, when differences in moisture were the greatest, there were no significant differences in kernel infection. At the last harvest date, when moisture levels were the same for resistant and susceptible hybrids, the differences in aflatoxin contamination were the greatest.

Researchers have conducted numerous studies in efforts to identify field resistance to *A. flavus* (1,13,15,16). Resistance has been selected using kernel infection and also by monitoring aflatoxin concentrations, or by selecting for resistance by evaluating for both parameters. Progress has been made in identifying genotypes with resistance to kernel infection (15,16). However, resistant material still allowed the accumulation of aflatoxin as determined in our study. Aflatoxin concentrations found in all of the resistant hybrids on the first harvest date were above the FDA tolerance level (20 ng/g). The only hybrid with an aflatoxin level below the tolerance level was Mp420 × Tx601 42 days after mid silk. In general, aflatoxin concentrations tend to be higher in ears mechanically inoculated with *A. flavus* than in ears naturally infected by *A. flavus*.

Temporal aflatoxin accumulation in resistant hybrids was similar to the pattern seen in susceptible hybrids; however, aflatoxin accumulation was at a much lower level in the resistant hybrids. Differences between the susceptible and resistant hybrids in kernel infection and aflatoxin contamination were detected at the early harvest dates and would allow for early selection of resistance in certain cases. However, the maximum differences in aflatoxin contamination were not found until later in the test. Kernel infection peaked 49 days after mid silk; whereas aflatoxin levels did not peak until the last harvest date. Since measuring aflatoxin concentrations is more definitive than kernel infection in selecting for resistance, field plots should be harvested near 63 days after mid silk.

Fig. 2. Grain moisture for susceptible (Mp307 × Mp428, GA209 × Mp339, and Mp68:616 × SC212M) and resistant (Mo18W × Mp313E, SC54 × SC76, and Mp420 × Tx601) maize hybrids at five harvest dates after mid silk.

Table 2. Aflatoxin accumulation (ng/g) in the grain of resistant and susceptible maize hybrids infected with *Aspergillus flavus*

Hybrid	Days after mid silk				
	35	42	49	56	63
Resistant					
Mo18W × Mp313E	34 ^a	33	65	53	93
Mp420 × Tx601	26	14	49	72	104
SC54 × SC76	36	24	48	139	186
Susceptible					
GA209 × Mp339	97	94	196	329	379
Mp307 × Mp428	89	134	290	183	510
Mp68:616 × SC212M	39	74	136	339	369
LSD ($P = 0.05$)	42	86	128	162	261

^a Data are mean values of 12 replicates.

LITERATURE CITED

1. Campbell, K. W., and White, D. G. 1995. Evaluation of corn genotypes for resistance to *Aspergillus* ear rot, kernel infection, and aflatoxin production. *Plant Dis.* 79:1039-1045.
2. Diener, U. L. 1989. Preharvest aflatoxin contamination of peanuts, corn, and cottonseed: A review. *Biodeterioration Res.* 2:217-244.
3. Gourama, H., and Bullerman, L. B. 1995. *Aspergillus flavus* and *Aspergillus parasiticus*: Aflatoxigenic fungi of concern in foods and feeds: A review. *J. Food Prot.* 58:1395-1404.
4. Gray, F. A., Faw, W. F., and Boutwell, J. L. 1982. The 1977 corn-aflatoxin epiphytotic in

- Alabama. Plant Dis. 66:221-222.
5. Guo, B. Z., Russin, J. S., Cleveland, T. E., Brown, R. L., and Damann, K. E. Evidence for cutinase production by *Aspergillus flavus* and its possible role in infection of corn kernels. Phytopathology 86:824-829.
6. Guo, B. Z., Russin, J. S., Cleveland, T. E., Brown, R. L., and Widstrom, N. W. 1995. Wax and cutin layers in maize kernels associated with resistance to aflatoxin production by *Aspergillus flavus*. J. Food Prot. 58:296-300.
7. Lillehoj, E. B., Kwolek, W. F., Vandegraft, E. E., Zuber, M. S., Calvert, O. H., Widstrom, N., Futrell, M. C., and Bockholt, A. J. 1975. Aflatoxin production in *Aspergillus flavus* inoculated ears of corn grown at diverse locations. Crop Sci. 15:267-270.
8. Lillehoj, E. B., Kwolek, W. F., Zuber, M. S., Bockholt, A. J., Calvert, O. H., Findley, R., Guthrie, W. D., Horner, E. S., Josephson, L. M., King, S., Manwiller, A., Sauer, D. B., Thompson, D., Turner, M., and Widstrom, N. W. 1980. Aflatoxin in corn before harvest: Interaction of hybrids and locations. Crop Sci. 20:731-734.
9. Lillehoj, E. B., Kwolek, W. F., Zuber, M. S., Horner, E. S., Widstrom, N. W., Guthrie, W. D., Turner, M., Sauer, D. B., Findley, W. R., Manwiller, A., and Josephson, L. M. 1980. Variation between hybrids developed for specific regions but grown at diverse locations. Plant Soil 54:469-475.
10. McMillian, W. W., Wilson, D. M., and Widstrom, N. W. 1985. Aflatoxin contamination of preharvest corn in Georgia: A six-year study of insect damage and visible *Aspergillus flavus*. J. Environ. Qual. 14:200-202.
11. Payne, G. A. 1992. Aflatoxin in maize. Crit. Rev. Plant Sci. 10:423-440.
12. Payne, G. A., Hagler, W. M., Jr., and Adkins, C. R. 1988. Aflatoxin accumulation in inoculated ears of field-grown maize. Plant Dis. 72:422-424.
13. Scott, G. E., and Zummo, N. 1988. Sources of resistance in maize to kernel infection by *Aspergillus flavus* in the field. Crop Sci. 28:504-507.
14. Scott, G. E., and Zummo, N. 1990. Preharvest kernel infection by *Aspergillus flavus* for resistant and susceptible maize hybrids. Crop Sci. 30:381-383.
15. Scott, G. E., and Zummo, N. 1990. Registration of Mp313E parental line of maize. Crop Sci. 30:1378.
16. Scott, G. E., and Zummo, N. 1992. Registration of Mp420 germplasm line of maize. Crop Sci. 32:1296.
17. Shane, S. M. 1994. Economic issues associated with aflatoxins. Pages 513-527 in: The Toxicology of Aflatoxins: Human Health, Veterinary, and Agricultural Significance. D. L. Eaton and J. D. Groopman, eds. Academic Press, New York.
18. Thompson, D. L., Payne, G. A., Lillehoj, E. B., and Zuber, M. S. 1983. Early appearance of aflatoxin in developing corn kernels after inoculation with *Aspergillus flavus*. Plant Dis. 67:1321-1322.
19. Widstrom, N. W., Wilson, D. M., and McMillian, W. W. 1981. Aflatoxin contamination of preharvest corn as influenced by timing and method of inoculation. Appl. Environ. Microbiol. 42:249-251.
20. Widstrom, N. W., Wilson, D. M., and McMillian, W. W. 1986. Differentiation of maize genotypes for aflatoxin concentration in developing kernels. Crop Sci. 26:935-937.
21. Zuber, M. S., Calvert, O. H., Lillehoj, E. B., and Kwolek, W. F. 1976. Preharvest development of aflatoxin B₁ in corn in the United States. Phytopathology 66:1120-1121.
22. Zummo, N., and Scott, G. E. 1989. Evaluation of field inoculation techniques for screening maize genotypes against kernel infection by *Aspergillus flavus* in Mississippi. Plant Dis. 73:313-316.