Effect of Four Inoculation Techniques on Infection and Aflatoxin Concentration of Resistant and Susceptible Corn Hybrids Inoculated with *Aspergillus flavus*

D. H. Tucker, Jr., L. E. Trevathan, S. B. King, and G. E. Scott

Graduate research assistant and associate professor, Department of Plant Pathology and Weed Science, Mississippi State University; and research plant pathologist and research agronomist, Agricultural Research Service, U.S. Department of Agriculture, Mississippi State 39762.

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Present address of senior author: Cooperative Extension Service, USDA, University of Georgia, College of Agriculture, Athens 30602; present address of third author: ICRISAT, Patancheru P.O., Andhra Pradesh 502 324, India.

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ABSTRACT

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Aflatoxin concentration of kernels of field-grown corn hybrids was determined after using four inoculation techniques to inoculate the ears with *Aspergillus flavus*. In addition, bright greenish-yellow fluorescence, visible growth of *A. flavus* on kernels, insect damage to ears, and kernel colonization by *A. flavus* were determined and evaluated as predictive estimates of aflatoxin concentration. Total aflatoxin concentration of hybrids was higher in kernels that received pinbar, knife, and exposed-kernel inoculations than in silk-inoculated or uninoculated kernels. Only the pinbar technique permitted complete separation of hybrids into groups based on the relative susceptibility to kernel infection by *A. flavus*. Percentage of kernels containing *A. flavus* was significantly correlated with total aflatoxin concentration. Thus, measurement of kernel infection frequency appears to be a valid predictive estimate for aflatoxin concentration in corn.

Additional key words: disease resistance, maize, *Zea mays*.

Recent research has been directed toward development of resistant corn hybrids as a means of controlling infection by *Aspergillus flavus* (Lk. ex Fr.) and subsequent aflatoxin formation. Although resistance has not been well documented (8), several researchers have noted that aflatoxin levels are genetically influenced (12,14,15,17,19,22). By compiling data from several sources, Widstrom and Zuber (21) found significant differences in aflatoxin levels among hybrids in a majority of studies.

Since aflatoxin levels are often low and quite variable, several inoculation techniques have been used by researchers to evaluate susceptibility to *A. flavus* and to aflatoxin production. As a result, few studies involving corn hybrid comparisons have utilized the same method of inoculation. King and Scott (7) tested four inoculation techniques for infection of corn hybrids by *A. flavus*; two involved mechanical damage to kernels and two did not. Both kernel damage treatments resulted in infection rates that differed significantly among hybrids, while infection rates for treatments without mechanical kernel damage were not different. Widstrom et al (20) tested three inoculation techniques but failed to find differences among hybrids for aflatoxin contamination, insect damage to the ear, or percentage of ears with visible *A. flavus*. It is possible that use of different inoculation techniques in studies of corn hybrid response to *A. flavus* could account for some of the conflicting reports of resistance or susceptibility of a particular hybrid.

The objective of this study was to evaluate the effectiveness of four inoculation techniques for causing infection by *A. flavus* and the subsequent aflatoxin concentration of the kernels of corn hybrids that differ in susceptibility to the fungus.

MATERIALS AND METHODS

Based on data (G. E. Scott and S. B. King, *unpublished*) from pinbar inoculation tests in 1980 and 1981, four single crosses were selected for this study. These crosses (Mp428 × SC212M, GT106 × SC343, Mol8W × Mp412, and Mp490 × Tx601) averaged 48, 35, 5, and 6%, respectively, kernel infection by *A. flavus*. The first two single crosses were selected as susceptible and the latter two as resistant.

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The hybrids were planted on 12 and 30 April in 1982 and 1983, respectively. Thirty-five kernels were planted in 5-m, single-row plots, and the stand was thinned to 15–20 evenly spaced plants per plot. A randomized complete block design with four hybrids and five treatments (inoculation techniques) was used with three replications.

Inoculum was derived from isolate NRRL 3357 of *A. flavus* (obtained from E. B. Lillehoj, Southern Regional Research Center, U.S. Department of Agriculture, New Orleans, LA) which is known to produce aflatoxin on corn. Inoculum was increased by placing conidia on 15 ml of Czapek's solution agar and growing the resulting cultures for 3 wk at 28 C. Conidia in each plate were washed from the agar with 20 ml of sterile, distilled water amended with two drops of Tween-20 per liter, filtered through double-layered cheesecloth, and stored at 4 C for 3 wk.

Treatments consisted of the different inoculation techniques and an uninoculated control. Silks were inoculated 6–7 days after midsilk (50% of primary ears in a plot showing silks) or when silks were yellow-brown (6). A conidial suspension containing 10^7 conidia per milliliter was atomized (0.7 ml per ear) onto the silks of all primary ears in a plot. The silks and upper part of the developing ear were covered with a plastic bag (secured by a rubber band), and the entire ear was enclosed in a pollinating bag (6). After 4 days, the plastic bag was removed, but the pollinating bag remained in place until harvest.

The exposed-kernel, pinbar, and knife inoculations were made on primary ears only, all at 20 days after midsilk. In the exposed-kernel inoculation, husks were pulled from one side of the ears to expose approximately 25% of the ear surface (7). A conidial suspension of 10^7 conidia per milliliter was atomized (1.4 ml per ear) onto the exposed kernels. The husk was repositioned and secured by rubber bands, and the inoculated ear was covered with a pollinating bag until harvest (7).

For pinbar inoculations, a plastic resin bar was cast with 35 sewing needles in a single row. Approximately 6 mm of the pointed ends protruded from the bar, and the needles were spaced to permit inoculation of successive kernels within a row. The needles were dipped into a conidial suspension of 2×10^7 conidia per milliliter, and the bar was aligned parallel with the ear axis. The needles were then pushed through the husk and into the underlying kernels (7).

In knife inoculations, the tip of a paring knife was dipped into a conidial suspension of 2×10^7 conidia per milliliter. The knife tip was then plunged through the husk and into the underlying kernels (two to three kernels wounded) in the lower one-third of the ear (20).

All plots were harvested by hand 60 days after the midsilk date. Harvested ears were bagged in the field after husks were removed, then placed into a forced-air drier at 42 C for 7 days to dry the grain below 13% moisture. All harvested ears, except those that had been pinbar-inoculated, were rated for visible growth of *A. flavus* on a scale of 1 (no visible growth) to 6 (100 kernels or more with visible growth at the ear tip and side). The same samples were also rated for insect damage to the ear on a scale of 1 (no insect damage) to 6 (extensive insect damage). Whole ears were mechanically shelled, and a random sample of 260 kernels was collected for determination of percent infection. Remaining kernels were bulked and stored at 4 C until aflatoxin analysis.

Infection by *A. flavus* was determined by plating 260-kernel samples on Czapek's solution agar amended with 7.5% NaCl. Kernels were incubated for 1 wk at 28 C, and the percentage of kernels containing *A. flavus* was determined.

Kernel rows one and two on both sides of the pinbar inoculated row were removed by hand and placed in separate containers. A subsample of 100 kernels representing each row position was plated to determine percent infection by *A. flavus*. The remaining kernels were stored at 4 C until further analysis.

Aflatoxin concentration and percentage of kernels exhibiting bright greenish-yellow fluorescence (BGYF) were determined from a random sample of bulked grain (1 kg) from each plot. A 260-kernel subsample was removed from each bulked sample and observed under longwave UV light to determine percentage of BGYF. Both sides of each kernel were examined for fluorescence. Subsamples were then returned to the 1-kg sample, and bulked samples were ground twice to a particle size of 1 mm for aflatoxin extraction. Fifty-gram subsamples were removed from this material and frozen until subsequent aflatoxin extraction. Aflatoxin extraction and quantitation were accomplished by the procedure of Thean et al (16).

RESULTS

Considering all hybrids, the aflatoxin concentration was highest when the exposed kernel inoculation technique was used (Table 1). The aflatoxin concentration of kernels was similar in those from row one inoculated by using the pinbar and knife-inoculation techniques, but both were significantly greater than concentrations in those from row two inoculated by the pinbar, silk, and the uninoculated control treatments. Kernel infection by *A. flavus* was significantly greater for row one of the pinbar-inoculated and exposed-kernel inoculation technique and lowest for the silk inoculation. Also, row one of the pinbar-inoculated and exposed-kernel inoculation techniques had a higher percentage of kernels with BGYF than were found with the other inoculation techniques. The exposed-kernel inoculation technique resulted in ratings for visible growth of *A. flavus* of 2.2 compared to an average rating of 1.1 for the knife, silk, and uninoculated check (data not included in Table 1 because differences among hybrids were not detected). Likewise, ratings for visible insect damage were significantly greater (2.4) for the exposed-kernel inoculation than the average for the other three treatments (1.8), but differences among hybrids were not significant.

Considering all inoculation techniques, the two resistant hybrids (Mol8W × Mp412 and Mp490 × Tx601) had significantly lower aflatoxin concentrations and percentage of kernels infected with *A. flavus* than did the other two hybrids (Table 1). The average aflatoxin concentration and kernel infection of the susceptible hybrids by *A. flavus* exceeded that of the resistant hybrids by four and three times, respectively. Row-one kernels of both resistant hybrids had lower total aflatoxin concentration than the susceptible hybrids when inoculated with the pinbar. Kernel infection by *A. flavus* was also lower in resistant plants when the pinbar and exposed-kernel treatments were used. In addition, BGYF differed for the two types of hybrids with the exposed-kernel treatment.

Total aflatoxin concentration was significantly correlated with kernel infection regardless of the inoculation technique used (Table 2). Total aflatoxin concentration and BGYF were significantly correlated when the exposed-kernel technique was used or when the kernels on the row adjacent to the pinbar-inoculated row were assayed. Visible growth of *A. flavus* was correlated with total aflatoxin concentration only when the exposed-kernel inoculation technique was used. Insect damage was not correlated with total aflatoxin concentration.

DISCUSSION

Aflatoxin concentrations were high enough to identify differences among corn hybrids with the pinbar-row-one, knife, and exposed-kernel inoculations. Separation of the susceptible and resistant hybrids into separate groups for aflatoxin concentration was complete for the pinbar-row-one treatment, but there was some overlapping between groups with the knife- and exposed-kernel inoculation techniques. Samples evaluated for aflatoxin from the knife- and exposed-kernel inoculation techniques contained varying amounts of wounded (knife) kernels and insect-damaged kernels (exposed kernels) which likely would have much higher aflatoxin concentration than undamaged kernels. The possible contributory role of insects to aflatoxin contamination of corn has been studied (3,4,9–11). Although ears inoculated with a pinbar were not rated for insect damage, little or none was noted on kernels removed for analysis.

Various in vitro techniques have been used in attempts to screen corn hybrids for resistance to *A. flavus* and subsequent aflatoxin contamination (1,12,18). Although differences among hybrids have

TABLE 1. Total aflatoxin concentration, percentage of infected kernels, and percentage of bright greenish-yellow fluorescent kernels of four field-grown corn hybrids inoculated with *Aspergillus flavus* at Starkville, MS, in 1982 and 1983

Hybrid	Inoculation technique						
	Pinbar-1	Pinbar-2	Knife	Exposed kernel	Silk	Uninoculated	Mean[w]
Aflatoxin concentration (total ng/g)[x]							
Mp428 × SC212M	233 a[y]	31 a	104 a	500 ab	1 b	6 a	41 a
GT106 × SC343	690 a	1 bc	104 a	911 a	9 a	1 b	37 a
Mo18W × Mp412	9 c	0 c	25 b	244 bc	1 b	1 b	6 b
Mp490 × Tx601	30 b	3 b	44 ab	143 c	0 b	6 a	13 b
Mean	80 B[z]	3 C	61 B	362 A	2 C	3 C	
Kernel infection (%)							
Mp428 × SC212M	15.3 a	7.5 a	3.2 b	10.3 b	0.2 a	2.6 a	5.6 a
GT106 × SC343	18.6 a	6.3 a	7.9 a	17.0 a	1.1 a	1.2 ab	7.2 a
Mo18W × Mp412	2.2 c	2.1 b	3.2 b	3.6 c	0.7 a	0.3 b	1.7 b
Mp490 × Tx601	9.2 b	1.4 b	1.8 b	6.4 c	0.6 a	1.2 ab	2.7 b
Mean	10.3 A[z]	3.7 B	3.8 B	7.9 A	0.6 C	1.3 BC	
Bright greenish-yellow fluorescent kernels (%)							
Mp428 × SC212M	1.9 b	0.5 a	0.4 a	3.3 a	0.1 a	0.6 a	0.9 a
GT106 × SC343	5.8 a	0.6 a	0.7 a	4.5 a	0.2 a	0.1 a	1.6 a
Mo18W × Mp412	0.7 c	0.0 a	0.7 a	1.6 b	0.1 a	0.0 a	0.6 a
Mp490 × Tx601	3.3 b	0.0 a	0.6 a	1.6 b	0.0 a	0.0 a	0.8 a
Mean	2.7 A[z]	0.2 B	0.6 B	2.7 A	0.1 B	0.1 B	

[w] Means across inoculation techniques were retransformed to original measurements.
[x] Data were transformed logarithmically for statistical analysis and means are antilogs.
[y] Means within a column for each response variable not followed by the same letter differ significantly ($P = 0.05$) from each other.
[z] Means within a row not followed by the same uppercase letter differ significantly ($P = 0.05$) from each other.

TABLE 2. Correlation coefficients comparing total aflatoxin concentration with fungal growth, insect damage, bright greenish-yellow fluorescence (BGYF), and percentage of infected kernels of corn inoculated with *Aspergillus flavus* by using five inoculation techniques

Inoculation technique	Correlation[a] between total aflatoxin and:			
	Visible *A. flavus* growth	Visible insect damage	BGYF of kernels	*A. flavus* kernel infection
Pinbar - 1	...[b]	...	0.55**	0.61**
Pinbar - 2	...	...	0.03	0.62**
Knife	−0.21	0.23	0.11	0.41*
Exposed kernel	0.56**	0.29	0.91**	0.88**
Silk	0.11	0.34	0.01	0.83**
Uninoculated control	0.18	0.09	0.07	0.07

[a] Asterisks * and ** indicate statistical significance at $P = 0.05$ and $P = 0.01$, respectively.
[b] Not applicable to this inoculation technique.

been reported, results were frequently not repeatable in vivo or there was no correlation between infection and aflatoxin concentration. Rating for visible growth of *A. flavus*, insect damage, percent BGYF, or infection by *A. flavus* would require less sophistication and expense than extraction and quantification of aflatoxin. Eliminating the need for high-pressure liquid chromatography or thin-layer chromatography for determining aflatoxin concentration would be helpful in corn hybrid screening programs. Percentage of kernel infection was the only parameter measured that was consistently correlated with total aflatoxin (Table 2).

BGYF of corn kernels has been used as a presumptive technique for aflatoxin comtamination, and Dickens and Whitaker (2) have demonstrated that kernels with BGYF have a much higher aflatoxin level than the non-BGYF kernels. In the present study, BGYF was only correlated with total aflatoxin for two of the inoculation methods. Although detectable levels of aflatoxin were present in each sample positive for BGYF, several false-negative samples were present; i.e., there was no fluorescence but aflatoxin was present. Fully 37% of kernel samples containing less than 1% BGYF contained at least 20 ng of aflatoxin per gram of kernel tissue, and levels as high as 500 ng/g were detected. At the present time, selecting for low-BGYF may not be a substitute for selecting for reduced levels of aflatoxin. The lack of significant correlations for insect damage, and growth of *A. flavus* with total aflatoxin means that neither insect damage nor visible growth of the fungus alone would likely be reliable predictions for aflatoxin concentrations of corn hybrids.

Higher aflatoxin levels were recorded in 1983 than 1982. Rainfall amounts from 1 July through 15 August were 26.4 and 9.6 cm for 1982 and 1983, respectively. These results are in agreement with previous reports that moisture stress conditions are conducive to aflatoxin contamination in corn (5,13). Low infection rates have been measured in four consecutive growing seasons for hybrids Mol8W × Mp412 and Mp490 × Tx601 when artificially inoculated. These hybrids are resistant to infection by *A. flavus* and subsequent aflatoxin concentration, even under environmentally diverse growing conditions.

Use of the pinbar inoculation technique, and aflatoxin analysis of the adjacent row of kernels parallel to the inoculated row, resulted in statistical differentiation of the relative susceptibility of corn hybrids to *A. flavus*. Quantification of infection rates by *A. flavus* appears to be a reliable method for predicting relative aflatoxin concentration. Therefore, use of pinbar-row-one inoculation and evaluation for kernel infection by *A. flavus* seems to be a reliable method for identifying corn genotypes differing in amounts of aflatoxin concentration. Use of BGYF as a presumptive aflatoxin detection method may not be as precise as necessary for screening corn genotypes.

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