Aflatoxin B_1 Production in an Eight-Line Diallel of Zea mays infected with Aspergillus flavus

M. S. Zuber, O. H. Calvert, W. F. Kwolek
E. B. Lillehoj, and M. S. Kang

Formerly Supervisory Research Agronomist, Cereal Genetics Research Unit, U.S. Department of Agriculture, retired and now Professor of Agronomy; Professor of Plant Pathology, University of Missouri Agricultural Experiment Station, Columbia, MO 65201; Biometrician; Research Microbiologist, Northern Regional Research Center, Federal Research, Science and Education Administration (SEA), U.S. Department of Agriculture, Peoria, IL 61604; and former Graduate Assistant in Agronomy, University of Missouri Agricultural Experiment Station, Columbia, MO 65201, now a Plant Breeder, Cargill Seed Company, St. Peter, MN 56082.

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ABSTRACT

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Wide differences in aflatoxin B_1 produced in corn grain were found among 28 possible single crosses from eight randomly-selected inbred lines used as parents. Ears were artificially inoculated with conidia of *Aspergillus flavus*. In a diallel analysis of the aflatoxin B_1 data, highly significant general combining ability (GCA) effects were found but the specific combining ability (SCA) effects were found to be nonsignificant. The results suggest that the levels of aflatoxin B_1 observed in corn infected with *A. flavus* were under genetic control. Lower concentrations of aflatoxin B_1 in grain were associated with the inbred lines H60 and Mo17 and higher levels with Oh545 in crosses with inbred lines N104, N7B, N28, H84, and Mo5. These findings suggest a cyclic selection program should be effective in developing corn lines with resistance to aflatoxin contamination.

Additional key words: corn, diallel analysis.

Aflatoxin B_1 is produced as a secondary metabolite (5) by *Aspergillus flavus* Link ex Fries growing on corn grain (*Zea mays* L.) both before and after harvest (1, 3, 6, 9, 10, 11, 12). This mycotoxin is a powerful carcinogen in animals that ingest contaminated corn and can, at low levels, dramatically reduce feed efficiency and general health (5). Corn constitutes a major part of the human diet in many countries. Therefore, aflatoxin B_1 contamination of corn also may deleteriously affect the health and well-being of humans (5). Despite these findings, little research has been conducted on the genetic control of aflatoxin B_1 in corn after *A. flavus* colonization. Whether the levels of aflatoxin B_1 can be controlled genetically by the host has not been established definitely, but limited studies involving *A. flavus* and several genotypes of corn showed significant differences among them for aflatoxin B_1 levels (4, 6, 8). No heritability studies are known involving other nonpathogenic fungus-plant associations. The objective of this study was to test the hypothesis that differences exist in levels of aflatoxin B_1 produced on grain from single crosses among corn belt inbred lines infected with *A. flavus*.

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MATERIALS AND METHODS

In 1976 a field experiment was conducted in a randomized complete block design with all of the possible 28 single crosses and their reciprocals among eight inbred corn belt line parents (H60, H84, Mo5, Mo17, N7B, N28, N104, Oh545). The eight inbred lines were considered as being randomly selected as we had no previous knowledge of the amounts of aflatoxin B_1 that would be produced in association with *A. flavus*. Seeds from the 56 single crosses were planted near Columbia, Missouri, in plots each consisting of a single row of 13 plants that were spaced 33 cm in the row and 96 cm between the rows. Two replications were used.

Husks of the primary ears were pulled back to expose the developing kernels 20 days after 50% or more of the ears in a plot had visible silks. The kernels were injured with a pinboard composed of 85 sewing pins arranged in five rows of 17 pins covering an area of 25 mm × 102 mm with 0.5 cm of the sharp ends extending beyond the board.

The inoculum was prepared from suspensions of conidia of *A. flavus* (NRRL 3357) grown on potato-dextrose agar in petri dishes for 14 days at 28 C. Conidia were washed from the surface of the agar with distilled water containing 0.01% Triton-X (Rohm and Haas Co., Philadelphia, PA 19105). The resulting suspension was

adjusted to 2×10^7 conidia/ml.

Approximately 1.5 ml of the conidial suspension was atomized over the injured kernels with a Model 15 DeVilbiss atomizer (The DeVilbiss Co., Somerset, PA 15501), after which the husks were repositioned over the ear and secured with rubber bands. The ears were covered for seven days with plastic bags after inoculation to maintain a high humidity which is favorable for conidium germination. On the eighth day the plastic bags were removed and replaced with brown-paper tassel bags. Thirty days later the ears were harvested at approximately physiological maturity and dried at 60 C for four days to less than 13% moisture. Infected kernels from the inoculated areas of each ear were shelled and bulked by plots and then ground in a 30.5 cm Raymond hammer-mill with a screen having 3.2-mm perforations (Raymond Pulverizer Division, Combustion Engineering Co., Inc., 200 West Monroe, Chicago, IL 60606). Ground corn samples were assayed for aflatoxin B_1 as described in the official First Action of the Association of Official Analytical Chemists (2). Quantities of aflatoxin B_1 present in the extracts were determined on thin-layer chromatographic plates coated with 0.5 mm Adsorbosil-1 (Applied Science Lab. Inc., Box 440, State College, PA 16801). The plates were developed with chloroform:acetone:water (88:12:1.5, v/v), and the fluorescent zones were measured densitometrically. Aflatoxin B_1 levels were recorded as nanograms of aflatoxin B_1 per gram of corn grain. Since there was a wide range in aflatoxin B_1 produced in the individual plots (range: 767 to 11,101 ng/g), the means for the replications were computed geometrically, and analyses computed by log transformations of the data to achieve normality (13). Statistical analysis of the data was by Method three (model I submethod) of Griffing (7) which provided estimates of general combining ability (GCA), specific combining ability (SCA), and reciprocal effects using only single-cross data. The general linear model for the analysis was

$$x_{ijk} = \mu + b_k + g_i + g_j + s_{ij} + r_{ij} + e_{ijk}$$

in which x_{ijk} = observed value in ng/g, μ = mean, b_k = block effect, g_i = GCA effect of line i, g_j = GCA effect of line j, s_{ij} = SCA effect of ij cross, r_{ij} = reciprocal effect of lines i × j and j × i, and e_{ijk} = residual error effect (7).

RESULTS

Mean aflatoxin B_1 levels (ng/g) for all possible single-cross combinations derived from the eight parental lines are shown in Table 1. The replications means ranged from a low of 1,200 ng/g for N28 × H60 to a high of 8,678 ng/g for Oh545 × N28. Line means (means for inbreds used as both male and female parents) showed that inbred H60 had the lowest ng/g of aflatoxin B_1 while Oh545 had the highest.

The analysis of variance is shown in Table 2. The overall mean for the experiment was 3,693 ng/g. Aflatoxin B_1 levels were highly significant for the GCA effects but not significant for either SCA effects or reciprocal effects. Replication differences also were highly significant.

The GCA effects expressed logarithmically for each inbred line are shown in Table 3. Parental lines H60 and Mo17 had highly significant negative β values, and Oh545 had highly significant positive β values. Crosses made between the parents with low negative β values resulted in the production of significantly lower levels of aflatoxin B_1 and the crosses between these parents with higher positive

TABLE 1. The aflatoxin B_1 (ng/g) geometric means for the eight parental lines used as female and as male parents for 28 single crosses and 28 reciprocals infected by *Aspergillus flavus*

	H60		H84		Mo5		Mo17	
Parent	♀	♂	♀	♂	♀	♂	♀	♂
H60	...	...	4,866	3,183	2,701	3,284	1,280	1,514
H84	3,183	4,866	...	...	4,491	5,335	2,721	3,982
Mo5	3,284	2,701	5,335	4,491	...	...	6,074	4,166
Mo17	1,514	1,280	3,982	2,721	4,166	6.074	...	...
N7B	3,927	1,940	3,936	4,248	4,593	4,996	4,030	1,793
N28	1,652	1,200	6,430	2,213	3,870	4,236	1,481	2,940
N104	2,058	2,023	2,258	4,382	4,020	4,048	2,475	2,780
Oh545	4,480	4,701	5,024	8,442	4,109	6,373	3,772	5,389
Line mean[a]	2,490		4,227		4,344		2,851	

	N7B		N28		N104		Oh545	
Parent	♀	♂	♀	♂	♀	♂	♀	♂
H60	1,940	3,927	1,200	1,652	2,023	2,058	4,701	4,480
H84	4,248	3,936	2,213	6,430	4,382	2,258	8,442	5,024
Mo5	4,996	4,593	4,970	3,807	4,048	4,020	6,373	4,109
Mo17	1,793	4,030	2,940	1,481	2,780	2,475	5,389	3,772
N7B	...	...	6,691	6,135	2,941	3,304	3,188	3,397
N28	6,135	6,691	...	...	2,944	3,376	8,678	7,094
N104	3,304	2,941	3,376	2,944	...	...	7,814	3,939
Oh545	3,397	3,188	7,094	8,678	3,939	7,814	...	...
Line mean[a]	3,699		3,587		3,234		5,170	

[a]Line means were computed from the single crosses for each inbred used as both female and male parent.

GCA β values, resulted in relatively higher levels of aflatoxin B_1. Estimates of SCA and reciprocal effects were not included since they were generally of small magnitude (H84 × N28 was the only cross that was significant) and probably of minor consequence. However, there were two exceptions: the SCA effect for N7B × N28 was positive and significant but crosses H60 × N28 and N7B × Oh545 were negative and significant, and a reciprocal effect was positive and significant for the H84 × N28 cross. This latter exception represents one reciprocal cross out of 28.

The levels of aflatoxin B_1 for single crosses between the lowest (H60) and highest (Oh545) inbreds used as parents in the diallel are given in Table 4 for each of the other inbreds as parents. The mean of the lowest × low parental crosses (H60 × Mo17 and H60 × N104 and reciprocals) was not markedly different from the mean level of aflatoxin B_1 developed by the lowest × intermediate parent crosses (H60 × N7B and H60 × N28 and reciprocals). However, the mean of the lowest × high parental crosses (H60 × H84 and H60 × Mo5 and reciprocals) was markedly higher and nearly equal to the overall mean of the experiment. The line means for the highest × low parental crosses (Oh545 × Mo17 and Oh545 × N28 and reciprocals) and the highest × intermediate

TABLE 2. Analysis of variance for log transformations of aflatoxin B_1 levels (nanograms per gram of corn) for the 28 single crosses and 28 reciprocals between eight inbred corn lines used as female and as male parents infected with *Aspergillus flavus*

Source of variation	d. f.	Mean square[a]
Replicates	1	0.5961**[b]
General combining ability (GCA)	7	0.3361**
Specific combining ability (SCA)	20	0.0641
Reciprocals	28	0.0340
Error	55	0.0521

[a]Based on log of aflatoxin B_1 levels (nanograms per gram) of corn.
[b]Double asterisks indicate statistical significance, $P = 0.01$.

TABLE 3. General combining ability effects (GCA), their standard error (S.E.), and the aflatoxin B_1 (nanograms per gram of corn) estimates for the eight parental lines used as female and male parents in the 28 possible single crosses and 28 reciprocal crosses infected with *Aspergillus flavus*

Inbred line	Estimated effect[a] (logarithm)	Aflatoxin B_1 geometric mean[b] (ng/g)	Rank
H60	−0.1861**[c]	2,342	1
Mo17	−0.1175**	2,742	2
N104	−0.0535	3,178	3
N28	−0.0069	3,538	4
N7B	0.0151	3,722	5
H84	0.0687	4,211	6
Mo5	0.0958*[c]	4,482	7
Oh545	0.1842**	5,493	8

[a]Standard error = 0.0436.
[b]The grand mean x = 3,692.6 ng/g aflatoxin B_1 for the experiment.
[c]The asterisks, * and **, indicate statistical significance at $P = 0.05$ and $P = 0.01$, respectively.

TABLE 4. Geometric line means for the parents that developed the lowest (H60) and the highest (Oh545) levels (nanograms per gram of corn grain) of aflatoxin B_1 crossed with other parents in a diallel that ranked low (Mo17 and N104), intermediate (N25 and N7B), and high (H84 and Mo5) by general combining ability (GCA) effects estimates[a]

Corn line parents	Parent	Crossed with line	
		H60 (2,342)	Oh545 (5,493)
Low aflatoxin:			
Mo17 (2,742)	♀	1,280	3,772
	♂	1,514	5,389
N104 (3,178)	♀	2,023	3,939
	♂	2,058	7,814
Line mean		1,685	5,001
Intermediate aflatoxin:			
N28 (3,538)	♀	1,200	7,084
	♂	1,652	8,678
N7B (3,722)	♀	1,940	3,397
	♂	3,927	3,188
Line mean		1,971	5,081
High aflatoxin:			
H84 (4,211)	♀	4,866	5,024
	♂	3,183	8,442
Mo5 (4,482)	♀	2,701	4,109
	♂	3,284	6,373
Line mean		3,424	6,773

[a]Estimates of aflatoxin B_1 in nanograms per gram of grain for the eight parental inbred lines are given in parentheses.

(Oh545 × N7B and Oh545 × N28 and reciprocals) were still much higher. The highest geometric "line mean" for the highest × high parental crosses (Oh545 × H84 and Oh545 × Mo5 and reciprocals) was 5,773 ng/g aflatoxin B_1 or 29% more aflatoxin B_1 than the lowest × low parental crosses.

DISCUSSION

The results of this study indicated that the magnitude of aflatoxin B_1 levels in *Zea mays* infected by *A. flavus* was under genetic control. The magnitude of the GCA effects in relation to the size of the SCA estimates also suggested that heritability of aflatoxin B_1 production in corn was additive in nature. These findings suggest to us that a cyclic selection program in corn should be effective in minimizing the levels of aflatoxin B_1.

Since the eight inbred lines involved in this study were randomly selected, there may be other genotypes available that would provide even lower levels of aflatoxin B_1 in their crosses than the lines used in this study. Additional lines should be subjected to a diallel analysis and artificial infection by *A. flavus*.

Research on sampling technique and sample preparation is urgently needed. The variability in aflatoxin B_1 levels encountered in this study between replications indicated the urgency. Shotwell et al. (12) have suggested that analysis for aflatoxin B_1 should be conducted on individual ears or perhaps on individual kernels to provide information on the large variability associated with sampling error in corn.

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