Genetic Control of Aflatoxin Production in Maize

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ABSTRACT

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Twenty-one possible F_1 single crosses among seven inbred maize lines (*Zea mays*) were planted in 20 replicates of single-hill plots, and developing ears were inoculated with *Aspergillus flavus* by a pinboard technique. Inoculated kernels from each ear were assayed for aflatoxin by high-performance liquid chromatography. To evaluate the effect of the number of replicates on the standard error of the mean, we used the error mean square for 20 replicates and computed standard errors of the mean assuming 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 replicates. Relative efficiencies of changing r_1 to r_2 replicates were calculated. Comparison of various numbers of replicates versus standard error of the mean suggested that eight replicates would be the most efficient number for aflatoxin B_1 field studies. Reductions in the standard errors of the mean were not appreciable with more than 10 replicates because relative efficiencies were changed little from 10 to 12 to 14 replicates, whereas standard errors were reduced when the number of replicates was changed from 6 to 8 to 10 replications. In the diallel analyses, genotypic differences were significant for both aflatoxin B_1 and B_2, as was the variance associated with general and specific combining ability effects. Specific combining ability sum of square estimates accounted for about 65% of the genotype sum of squares. Coefficients of variation were very high, 92 and 90% for aflatoxin B_1 and B_2, respectively. The estimates of general combining ability effects and rankings of aflatoxin levels from crosses and parental line means were in good agreement with a previous study conducted at the University of Missouri, even though only inoculated kernels were analyzed in this study versus whole ears in the previous study.

Aflatoxin production by *Aspergillus flavus* Link ex Fries in food commodities is significant because of the extremely toxic properties of aflatoxin and because of the ubiquitous nature of the fungus. The fungus grows on maize (*Zea mays* L.) kernels both in the field and in storage.

Results of several studies suggest that production of aflatoxin B_1 in maize kernels infected with *A. flavus* is under genetic control of the plant (8,12). Zuber et al (10) found significant differences for aflatoxin B_1 content among the 28 possible F_1 crosses of eight maize inbred lines by the pinboard inoculation method. Estimates of general combining ability (GCA) were highly significant, and estimates of specific combining ability (SCA) were not significant. Their results agreed with those found in an earlier study (11). Estimates of GCA and SCA effects for aflatoxin levels reported in their study (10) may have been affected by ear size, because kernels from whole ears were ground and analyzed rather than only infected kernels. Zuber et al (10) used only two replicates per location. Other similar aflatoxin contamination studies (4,9,12) have found very high experimental variation. Zuber et al (12), for example, reported coefficients of variation exceeding 300%.

The objective of our study was to determine if the results obtained by Zuber et al (10) were repeatable when only inoculated kernels were analyzed and whether better estimates of genetic effects with respect to control of aflatoxin production could be obtained with a larger number of replicates.

MATERIALS AND METHODS

Seven inbred lines, H84, Mo5, Mo17, N7B, N28, N104, and H60, were crossed in a diallel mating design (2) to produce 21 possible F_1 crosses. Neither reciprocal crosses nor parents were included in the evaluation. The 21 crosses were grown in a randomized complete block design with 20 replicates at a single location. An individual plot consisted of a single hill spaced 91 cm from adjacent hills in both directions. Five seeds were planted per hill, and emerging plants were later thinned to three. Ears from two of the three plants were inoculated, and only one of the two was sampled for aflatoxin analysis.

Ears were inoculated with *A. flavus* 18 days after midflower by pulling down a portion of the husk and injuring four rows of about 10 kernels each with a pinboard. Wounded kernels were sprayed with an aqueous suspension of *A. flavus* containing about 25,000 conidia per milliliter. The pinboard had two rows of 18 metal pins covering an area 62 × 10 mm with 12 mm of the sharp ends protruding. Husks were replaced and secured with a rubber band. A paper bag was placed over the ear and left until physiological maturity. Inoculated ears were harvested, kept inside the bag, and dried at 60 C for 5 days. Inoculated kernels were removed and ground in an Ika-Werk blender-grinder at the University of Missouri's Sinclair Research Center P-3 containment facility. The ground samples were analyzed by the following procedure developed by L. L. Wall of the Missouri Agricultural Experiment Station Chemistry Laboratory. The procedure is a scaled-down modification of the Association of Official Analytical Chemists method using chloroform (1).

Extraction was accomplished by placing 1 g of ground sample into a 25-ml centrifuge tube. One milliliter of water was added, making sure the sample was wetted, then 10 ml of chloroform was added. The tube was capped and shaken for 30 min. Tubes were then spun at maximum speed in a clinical centrifuge for at least 10 min. After carefully removing the tubes from the centrifuge, a 5-ml aliquot was withdrawn from the chloroform layer and placed in glass specimen bottles 22 mm in diameter × 39 mm high. The specimen bottles were warmed at low heat on a hotplate and the extract taken to dryness under N_2 purge. The dried extract was stored in a freezer until cleanup.

Sample extract cleanup on a small silica column and high-performance

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liquid chromatography (HPLC) analyses were done using a combination of procedures based on the work of Thean et al (8), Pons (6), and Panalaks and Scott (5). The dried extract was dissolved in 0.5 ml of chloroform with a Branston Instruments ultrasonic cleaner, and 0.5 ml of hexane was added. A cleanup column was made from a 146-mm Pasteur pipet containing 20 mm of Na_2SO_4 at the top, a 20-mm-thick layer of Porasil A silica, a 10-mm layer of Na_2SO_4, and a glass wool plug in the tip. The cleanup column was washed with 1–2 ml of hexane, leaving the hexane level at the top of the upper Na_2SO_4 layer. The sample was transferred from an Erlenmeyer flask to the column with a Pasteur pipet. One milliliter of chloroform and hexane (1:1, v/v) was added to rinse the flask and this wash was also added to the column. The solution was allowed to flow until reaching the level of the Na_2SO_4 layer. The column was then washed with 2 ml of hexane followed by 2 ml of anhydrous ethyl ether. Aflatoxin was eluted from the cleanup column with 3 ml of chloroform and methanol (97:1, v/v) into a clean, dry culture tube 16 × 76 mm. The sample was taken to dryness at 30 C under a stream of N_2, then stored in a freezer until analysis by HPLC. Amounts of B_1 and B_2 aflatoxin were determined using ultraviolet and fluorescence detection.

Determination of optimum number of replicates. The level of aflatoxin for the 20 replicates of each entry was analyzed as a randomized complete block, and the obtained error mean square was used to estimate the population error variance, σ_e^2. Standard errors of means with differing numbers of replicates were calculated as $SE_{\bar{x}_i} = s_e/\sqrt{r_i}$, where r_i varied from two to 20 replicates.

Relative efficiency (RE) of two combinations of replicates was calculated according to Steel and Torrie (7). Although usually used to compare different experimental designs, our application was to compare varying numbers of replicates.

Because different numbers of replicates were used in the standard error estimates, $s^2_{\bar{x}_1}$ and $s^2_{\bar{x}_2}$ were employed in place of s_1^2 and s_2^2 in the RE calculation. However, $s_1^2 = s_2^2 = \hat{\sigma}_e^2$ in this study, and the formula was reduced to RE of r_2 relative to $r_1 = [(n_2 + 1)(n_1 + 3)(r_2)]/[(n_1 + 1)(n_2 + 3)(r_1)]$, where n_j is the number of error degrees of freedom if r_j replicates are used and r_j is the number of replicates. Values greater than 1.0 indicate that r_2 is more efficient than r_1. REs may also be expressed as percentages to more clearly indicate differences, e.g., 1.20 = 120% or r_2 has a 20% advantage relative to r_1.

Diallel analyses. The linear model for the diallel analysis (2) was: $Y_{ij} = \mu + g_i + g_j + s_{ij} + r_k + e_{ijk}$, where Y_{ij} is the aflatoxin level of cross ij, μ is the population mean, g_i is the GCA effect for parent i, g_j is the GCA effect for parent j, s_{ij} is the SCA effect for the cross ij, r_k is the effect of the k^{th} replication, and e_{ijk} is the random error term associated with the cross ij in the k^{th} replication.

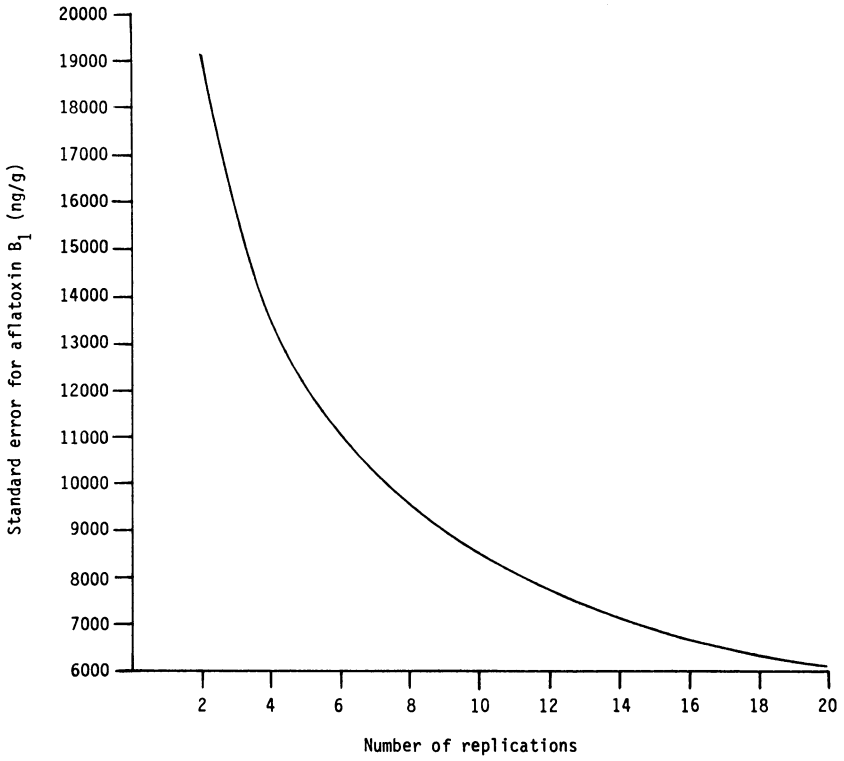

Fig. 1. Influence of increased replication on the standard error of the mean for aflatoxin B_1.

RESULTS AND DISCUSSION

Determination of optimum number of replicates. Error variance estimates from the 20-replicate analyses were 725,668,551 ng/g for aflatoxin B_1 and 1,333,525 ng/g for aflatoxin B_2. Means and coefficients of variation were 29,321 ng/g and 92% for B_1 and 1,273 ng/g and 90% for B_2, respectively.

Emphasis in the laboratory analyses was on aflatoxin B_1; results for B_2 are probably not of the same degree of reliability. Generally, higher levels of aflatoxin B_2 are associated with higher B_1 levels; this was the case in this study.

Figure 1 shows the relation between numbers of replicates and standard errors of the mean for aflatoxin B_1. The greatest reduction in the standard errors of the means occurred in the area of the curves from two to eight replicates. Beyond this, the rate of decrease in the standard errors of the means diminished rapidly. Eight replicates are recommended on the basis of this study and the environment under which it was grown. A reasonable compromise between price for additional replicates and the diminishing reduction in standard error of the mean is thus obtained.

Estimates of the RE of increasing the number of replicates from r_1 to r_2 in steps of two or four replicates are given in Table 1. Because the calculated RE is a function of only replicates and error degrees of freedom, the efficiencies apply equally to aflatoxin B_1 and B_2. Increases in RE exceeding 25% were found for each increase by two replicates up to 10 replicates. All increases by four replicates exceeded 25% increases in RE.

Table 1. Comparisons of relative efficiencies (RE) of using 2–20 and 4–20 replicates for analysis of aflatoxin in a 21-cross diallel with single-hill plots

Number of replicates		Percent RE
r_1	r_2	of r_2 vs. r_1
2	4	212
4	6	152
6	8	134
8	10	125
10	12	120
12	14	117
14	16	114
16	18	113
18	20	111
4	8	204
8	12	151
12	16	134
16	20	125

Although the experimental error was very high in this experiment, eight replicates should optimize the variation among entries. Fewer than eight replicates resulted in loss of precision, whereas numbers greater than eight resulted in little error reduction, and when the reduction in error is related to the cost of additional samples for analyses, gain per unit cost is poor.

Diallel analyses. Analyses of variance for aflatoxin B_1 and B_2 (Table 2) shows

that differences among genotypes were very highly significant ($P = 0.001$). GCA sums of squares were highly significant ($P = 0.01$) and made up 34 and 37% of the genotype sums of squares for aflatoxin B_1 and B_2, respectively. SCA sums of squares made up 66 and 63% of the genotype sums of squares for the same variables, respectively, and were also highly significant ($P = 0.01$). Thus, additive gene effects accounted for about one-third of the genetic variation, whereas dominance and epistatic effects accounted for about two-thirds. The high coefficients of variation (92 and 90% for B_1 and B_2, respectively) reflect the large experimental errors that have been found by us and others when determining aflatoxin production in field studies (4,12).

Parental line means calculated from the F_1 crosses that had that line as a common parent differed among each other for aflatoxin B_1 and B_2 (Tables 3 and 4). N104 and Mo17 had the lowest parental line means for aflatoxin B_1. The estimate of g_i for Mo17 was negative and differed significantly from zero (−7,708 ng/g). N7B had a positive significant g_i effect for aflatoxin B_1 (7,361 ng/g). For aflatoxin B_2, Mo17 had a significant negative g_i estimate (−371 ng/g) and N7B had a significant positive effect (330 ng/g).

Three estimates of s_{ij} effects were significant and negative for aflatoxin B_1 (Table 3): H84 × N7B (−10,763 ng/g), Mo17 × N7B (−12,690 ng/g), and Mo5 × N28 (−11,534 ng/g). Mo5 × N7B and N28 × N104 both had significant positive s_{ij} effects for aflatoxin B_1. For aflatoxin B_2, N7B was involved as a parent of one cross that had a significant negative s_{ij} effect and one that had a significant positive s_{ij} effect. H60 was the only parental line that had neither a significant g_i effect nor a significant s_{ij} effect.

Although additive variation existed, nonadditive variation made most of the total genetic variation in this study. These results do not agree with the findings of Zuber et al (10), where estimates of GCA were found to be much more important than estimates of SCA.

Relative rankings of the parental line means obtained in this study were in moderate agreement with those obtained by Zuber et al (10) (Table 3). Spearman's rank correlation coefficient for parental line means was 0.67 ($P > 0.05$). To further examine the data from the standpoint of repeatability of the Zuber et al (10) results, rankings of the F_1 crosses were compared (Table 5). Because whole ears were used in the 1978 study and only infected kernels were used in 1981, comparison of the means was not made. However, the rankings were compared by Spearman's rank correlation (7). The cross rank correlation was significant at

Table 2. Analyses of variance for aflatoxin B_1 and B_2

Source of variation	df	Mean squares[a] (ng/g) Aflatoxin B_1	Aflatoxin B_2
Replicates	19	789,813,605	1,316,247
Genotypes	20	1,909,782,451**	3,945,257
General combining ability	6	2,218,311,403*	4,910,383*
Specific combining ability	14	1,891,026,770*	3,616,525**
Error	329	725,668,551	1,333,525
Coefficient of variation (%)		92	90
Coefficient of determination		0.18	0.19

[a] Based on SAS Type III sums of squares, which were calculated because several cells were missing (3). * = Significant at $P = 0.01$ and ** = significant at $P = 0.001$.

Table 3. Estimates of general combining ability (GCA, g_i) and specific combining ability (SCA, s_{ij}) effects and parental line and cross means for aflatoxin B_1[a]

Inbred line parent	Inbred line parent (ng/g) H84	Mo5	Mo17	N7B	N28	N104	H60	Parental line Mean	Rank	1978 rank[b]
H84	*1,965*	3,337	1,463	−10,763*[c]	190	−2,514	8,286	30,794	5	6
Mo5	37,541	*2,876*	1,878	11,877*	−11,534*	−9,249	3,679	31,501	6	7
Mo17	24,915	26,691	*−7,708***	−12,690*	−4,247	9,247	4,347	26,901	2	2
N7B	27,634	51,056	16,047	*7,361***	7,654	−1,325	5,237	35,075	7	5
N28	32,169	19,794	16,976	44,350	*−29*	16,665**	−7,937	29,487	4	4
N104	23,379	18,678	26,284	29,656	44,244	*−4,493*	−3,841	25,574	1	3
H60	39,025	35,249	25,750	41,710	21,390	13,205	*26*	29,321	3	1

[a] GCA effects are on the diagonal (in italic), SCA effects are above the diagonal, and cross means are below the diagonal. $LSD_{0.05}$ $(g_i - g_j) = 7.467$, $LSD_{0.05}$ $(s_{ij} - s_{ik}) = 14{,}934$, $LSD_{0.05}$ $(s_{ij} - s_{kl}) = 12{,}933$, $LSD_{0.05}$ (cross mean$_i$ − cross mean$_j$) = 16,696, and $LSD_{0.05}$ (parental line mean$_i$ − parental line mean$_j$) = 6,816.
[b] Zuber et al (10).
[c] * = Significant at $P = 0.05$ and ** = significant at $P = 0.01$.

Table 4. Estimates of general combining ability (GCA, g_i) and specific combining ability (SCA, s_{ij}) effects and parental line and cross means for aflatoxin B_2[a]

Inbred line parent	Inbred line parent (ng/g) H84	Mo5	Mo17	N7B	N28	N104	H60	Parental line Mean	Rank
H84	*85*	181	94	−420	−34	−157	365	1,341	5
Mo5	1,670	*164*	94	447*[b]	−546*	−398	251	1,404	6
Mo17	1,075	1,177	*371***	−574**	−175	368	193	1,220	2
N7B	1,263	2,211	652	*330***	395	−31	181	1,537	7
N28	1,384	881	734	2,022	*21*	785***	−360	1,302	4
N104	972	849	1,069	1,321	1,914	*−202*	−218	1,091	1
H60	1,681	1,635	1,063	1,752	877	521	*−27*	1,255	3

[a] GCA effects are on the diagonal (italic), SCA effects are above the diagonal, and cross means are below the diagonal. $LSD_{0.05}$ $(g_i - g_j) = 320$, $LSD_{0.05}$ $(s_{ij} - s_{ik}) = 640$, $LSD_{0.05}$ $(s_{ij} - s_{kl}) = 554$, $LSD_{0.05}$ (cross mean$_i$ − cross mean$_j$) = 716, and $LSD_{0.05}$ (parental line mean$_i$ − parental line mean$_j$) = 292.
[b] * = Significant at $P = 0.05$, ** = significant at $P = 0.01$, and *** = significant at $P = 0.001$.

Table 5. Comparison of means and rankings for common single crosses in two diallel evaluations for aflatoxin B_1

Cross	1981 Mean aflatoxin B_1 (ng/g)	1981 Rank	1978[a] Mean aflatoxin B_1 (ng/g)	1978[a] Rank
N104 × H60	13,205	1	2,040	3
N7B × Mo17	16,047	2	2,912	6
N28 × Mo17	16,976	3	2,210	4
N104 × Mo5	18,678	4	4,043	14
N28 × Mo5	19,794	5	4,053	15
N28 × H60	21,309	6	1,426	2
N104 × H84	23,379	7	3,320	4
Mo17 × H84	24,915	8	3,352	12
Mo17 × H60	25,750	9	1,397	1
N104 × Mo17	26,284	10	2,628	5
Mo17 × Mo5	26,691	11	5,120	20
N7B × H84	27,634	12	4,092	16
N7B × N104	29,656	13	3,122	9
N28 × H84	32,169	14	4,322	17
Mo5 × H60	35,249	15	2,992	8
Mo5 × H84	37,541	16	4,913	19
H84 × H60	39,025	17	4,024	13
N7B × H60	41,710	18	2,933	7
N104 × N28	42,244	19	3,160	10
N28 × N7B	44,350	20	6,413	21
N7B × Mo5	51,056	21	4,794	18
Spearman's rank correlation coefficient = 0.45[b]				

[a]Zuber et al (10).
[b]Significant at $P = 0.05$.

$P = 0.05$ ($r_s = 0.45$). Although significant, the use of these results for predictability would be of little value. Lack of better agreement may be due in part to differential ear size effects in the Zuber et al (10) data. Given identical aflatoxin levels in the wounded kernels in each study, the dilution effects of ear size could result in substantial biases, upward for crosses with small ears and downward for crosses with large ears. Although the same basic extraction procedure was used, the analyses for the two experiments were done in different laboratories and separated by several years. In measuring the genetic control of aflatoxin production in maize, the results from this study suggest the inoculation method we used may not be optimal to estimate host response.

The general agreement of parental line and cross mean rankings between this study and that of Zuber et al (10) supports the hypothesis that genetic control of aflatoxin production in maize exists. Genetic variation per se must be shown before plant breeders would attempt to accumulate desirable alleles in breeding populations. The particular methodology employed is dependent on both the number of genes controlling expression and whether their effect is additive (shown by significant GCA variation) or nonadditive (shown by significant SCA variation). Progress expected from selection is related to the amount of variability available for selection and the accuracy with which that variation represents genetic differences (heritability). Results of this study and those conducted previously with similar genotypes suggest that inheritance appears quantitative in nature and that additive and some nonadditive variations are of significance. Because of the large amount of nongenetic variation present, a breeding procedure such as S_1 selection and conditions that maximize differences among genotypes would be recommended for selection of desirable genotypes.

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