Aflatoxin Accumulation in Developing Kernels of Eight Maize Single Crosses After Inoculation with *Aspergillus flavus*

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ABSTRACT

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Developing kernels of eight maize single crosses (*Zea mays*) were inoculated with *Aspergillus flavus* in four environments and assayed for aflatoxin. The eight crosses were from two A-inbreds crossed with four B-inbreds, as labeled for convenience. The two A-inbreds were about 88% related and there was little diffference between their aflatoxin means. Two of the B-inbreds averaged 22,168 and 17,915 ppb and had significantly less aflatoxin than the other two B-inbreds, which averaged 40,011 and 32,006 ppb. This study indicated that resistance to the accumulation of aflatoxin in developing kernels was under genetic control, that differences between duplicate assays were small, that variability among ears per plot was large, and that the greatest contribution to stable and economical estimates would come by increasing the number of replicates and keeping the number of ears to a low level of about five ears per plot.

Additional key words: corn, mycotoxin

Aflatoxin contamination of preharvest maize (*Zea mays* L.) caused by infection of developing kernels by *Aspergillus flavus* Link ex. Fr. continues to be a serious problem. Little progress has been made in identifying genotypes that could be used as dependable sources of resistance for attacking this problem by breeding. Characteristically, estimates of aflatoxin are variable, with large associated errors, and apparently are influenced by a complex of factors. This large variability dictates that attention be given to all experimental procedures, including the relative numbers of replicates, ears per plot, assays, sampling, etc., so that due consideration is given to the inherent variability of this character. Stable estimates are necessary to provide accurate appraisal of genotypes and initial confidence that the sources can be used successfully in breeding.

The purposes of this research were to determine the concentration of aflatoxin in developing kernels of eight maize single crosses in four environments after inoculation with *A. flavus* and to estimate variance components as considerations for improving experimental procedures.

MATERIALS AND METHODS

This study involved eight single crosses of maize grown in four environments (experiments 1–4). Kernels were inoculated by peeling back the husks on one side of the ear, applying a pinboard with a line of pins spaced 5 mm apart once to two adjacent rows to injure the kernels, and spraying about 0.5 ml of an *A. flavus* (NRRL3357) spore suspension containing 1×10^6 spores per milliliter on the injured area. Husks were repositioned, secured, and covered with plastic and paper bags to maintain humid conditions. After 3 days, the plastic bags were removed. The eight single crosses, which were dwarfs conditioned primarily by the recessive brachytic-2 gene (br-2), are listed in Table 1 and defined for convenience as crosses between two A-inbreds and four B-inbreds as parents. These dwarf types were chosen because their reduced height would fit within the height limitations of the rooms used in experiment 1.

Experiment 1 was conducted in controlled-environment rooms of the Southeastern Plant Environment Laboratories, Raleigh, NC (Phytotron). Lighting and other environmental aspects of the rooms have been described (2,5). The eight single crosses were chosen from among 24 crosses for their apparent range of aflatoxin levels. Plants were grown in plastic pots that had a volume of 4,000 ml. There were four single-plant

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Table 1. Mean aflatoxin levels in developing kernels of corn after inoculation with *Aspergillus flavus* in four environments (experiments 1–4)

	Aflatoxin levels (ppb)[a] for B-inbred parents in crosses with two A-inbred parents				
A-Inbred parents	HY375br (1)	A375br (2)	505Pbr (3)	HY41Pbr (4)	Mean
OH43br (1)	21,166	17,108	42,578	33,630	26,834
OH43VAbr (2)	23,218	18,761	37,600	30,460	26,576
Mean	22,168	17,915	40,011	32,006	26,705
LSR[b]					
Two-way array: 1.68					
A-Inbreds: 1.30					
B-Inbreds: 1.44					

[a] Values are aflatoxins $B_1 + B_2$; all means, including marginal means, were calculated from data transformed to logs, then retransformed to original scale.

[b] LSR : Means whose ratios, larger to smaller, exceed the LSR-values are significantly different ($P = 0.05$).

replicates for each cross, two in each of two rooms. Rooms were set at day/night temperatures of 30/26 C for 9/15 hr (with uninterrupted nights for a short day-length effect). Relative silking, inoculation, and harvest times were 51, 76, and 104 days after planting, respectively. All kernels of an ear, inoculated and uninoculated, were bulked for the assay sample. Assays were made at the USDA-ARS Southern Regional Research Center, New Orleans, LA.

Experiment 2 was grown in the field near Raleigh, NC, in 1981. The eight single crosses were among 54 crosses grown in a randomized complete block design of two replicates with 20 plants per plot. Six ears were inoculated per plot and five (approximately) were harvested together and shelled in bulk for the assay sample. Assays were made at the Mycotoxin Laboratory, North Carolina State University, Raleigh. Relative silking, inoculation, and harvest times were 68, 90, and 132 days after planting, respectively.

Experiments 3 and 4 were grown in the field in randomized complete blocks of eight replicates with 20 plants per plot in 1981 near Raleigh, NC, and Columbia, MO, respectively. Experiment 3 was handled on the same schedule as listed for experiment 2, with six ears inoculated and five (approximately) harvested and shelled in bulk. For experiment 4, an attempt was made to obtain 10 ears per plot, but the number ranged from 1 to 10, with an average of 3.9. Kernels showing visible growth of the fungus were classed infected and were separated from the uninfected and bulked for aflatoxin assay for each ear separately. Relative silking and inoculation times were 86 and 107 days after planting, respectively.

Kernels were ground, blended, and assayed for aflatoxin by the official first action method of the Association of Official Analytical Chemists (1). Quantities of aflatoxin B_1 and B_2 were determined on activated thin-layer chromatographic (TLC) plates coated with 0.5 mm of Absorbosil-1. Plates were developed with water-acetone-chloroform (1.5:12:88, v/v/v) in unequilibrated tanks, and fluorescent zones were measured densitometrically. Aflatoxin B_1 and B_2 were confirmed in representative positive samples by the formation of water adduct (1).

Relative proportions of aflatoxin B_1 and B_2 were 96 and 4%, respectively; consequently, data for the two types of aflatoxin were combined and called aflatoxin. Aflatoxin values were transformed to logarithms (base 10) for analyses of variance according to a mixed-effects model, with entry effects (single crosses) considered fixed. Mean estimates, including marginal means, were retransformed to the original scale of parts per billion for presentation in the tables. For comparing means, the least significant ratio (LSR) was calculated as the antilogarithm of the LSD of the transformed data (4).

The four experiments (each considered a separate environment) were examined for the relative importance of different sources of variation for aflatoxin accumulation by estimating the experimental error components of variance. The basic model assumes that experimental error is determined by three components: 1) aflatoxin determination error component, σ_d^2, which includes the laboratory error variance plus any variation due to subsampling the ground kernels; 2) an ear-to-ear, within-plot component of variance, $\sigma_{e(p)}^2$; and 3) a plot-to-plot experimental error component of variance, σ_s^2.

In cases where several ears were combined before a single subsample was taken for assay (experiments 1–3), it was assumed that combining and blending the material would have the same effect on the component $\sigma_{e(p)}^2$ as would simple averaging over the same number of ears. With these considerations, the variance of the difference between two single-cross means can be expressed as follows:

$$\sigma_{dif}^2 = 2(\sigma_d^2/dr + \sigma_{e(p)}^2/er + \sigma_s^2/r),$$

where r = number of replicates, e = total number of ears used in the assay in each plot, and d = total number of laboratory assays made in each plot. This formula is written to allow for d to be less than e, in which case ears are composited before the laboratory assays are made, or for d to be greater than e, in which case multiple laboratory assays are made on each ear.

Because duplicate assays had been made for 53 ears in experiment 4, it was the only one that allowed for estimation of all three components of variance. The determination error, σ_d^2, was estimated from the variation between duplicate determinations on these 53 ears. These duplicates were then averaged and used with the other single-ear values for the overall analysis; thus, the harmonic mean of "number of assays per ear" was 1.12. Transformed values (logs) for aflatoxin estimates were used for these analyses and are presented in these terms. Because the lower limit for detection of aflatoxin was 100 ppb, the transformation log (aflatoxin + 100) was used for aflatoxin values that had been recorded as 0.0 or trace.

RESULTS AND DISCUSSION

Means for aflatoxin levels over the four environments (experiments 1–4) are shown in Table 1 for the eight single crosses arranged in two-way arrays by A-inbreds and B-inbreds as parents. There was little difference between the two A-inbreds, which are related to each other about 88%. Two of the B-inbreds averaged 22,168 and 17,915 ppb and were significantly lower than the other two B-inbreds, which averaged 40,011 and 32,006 ppb, respectively. This difference between the first two and the last two B-inbreds as listed in Table 1 was detected in the first experiment and was a major reason for selecting these crosses for further investigation. These results indicate that resistance to aflatoxin accumulation is under genetic control; however, the levels for the two groups, about 20,000 and 36,000 ppb for the resistant and susceptible groups, respectively, may not be sufficiently different to indicate that the two resistant inbreds could serve as a practical source for genetic control of aflatoxin contamination. These results do provide encouragement, however, that breeding for resistance may be a viable approach. Widstrom and Zuber (6) have pointed out that aflatoxin accumulation is under genetic control but that differences among genotypes have been erratic.

Results from the components of variance considerations are presented in Table 2, but they were not consistent for the four experiments. For experiment 4, the assay determination error, $\hat{\sigma}_d^2$, is trivial compared with the other components ($\hat{\sigma}_d^2 = 0.010$, $\hat{\sigma}_e^2 = 0.283$, and $\hat{\sigma}_{e(p)}^2 = 0.135$), so that multiple laboratory determinations have little effect on increasing precision and there is little reason to use more than one determination per plot. This indicates that the current practice of compositing and

Table 2. Means, mean squares, and expected error mean squares from analyses of aflatoxin values after transformation to logs

Experiment	Mean	df	Mean square[a]	Expected mean square[b]
1	3.466	69	0.0455	$\sigma_d^2 + \sigma_{e(p)}^2 + \sigma_s^2$
2	4.207	53	0.0687	$\sigma_d^2 + \sigma_{e(p)}^2/n_o + \sigma_s^2$
3	4.869	49	0.0176	$\sigma_d^2 + \sigma_{e(p)}^2/n_o + \sigma_s^2$
4	4.350	44	0.8159	$\sigma_d^2/1.12 + \sigma_{e(p)}^2 + 3.8709\,\sigma_s^2$
4	···	184	0.2918	$\sigma_d^2/1.12 + \sigma_{e(p)}^2$

4 Component estimates: $\hat{\sigma}_d^2 = 0.010$; $\hat{\sigma}_{e(p)}^2 = 0.283$; $\hat{\sigma}_s^2 = 0.135$

[a] All mean squares are experimental error mean squares except 0.2918, which is a mean square for among ears in plots; in experiment 4, the mean squares are on a "per ear" basis but are on a "per plot" basis in experiments 1–3.

[b] Components are as follows: σ_d^2 = determination error, σ_e^2 = ear-to-ear within plot, and σ_s^2 = plot-to-plot experimental error; n_o is the harmonic mean of the number of ears per plot combined for each assay (about five).

blending ground kernels from several ears per plot and using a single sample for assay is satisfactory.

The next consideration is the number of ears per plot. Assuming one laboratory determination per plot, the determination component σ_d^2 becomes part of the plot error, $\hat{o}_d^2 + \hat{o}_s^2 = 0.145$. The ratio of variances $[\hat{o}_{e(p)}^2/(\hat{o}_d^2 + \hat{o}_s^2)] = (0.283/0.145) = 1.40$. The optimum number of ears per plot is $e = 1.40\,(c_1/c_2)$, where c_2 is the fixed cost per ear for producing a given number of plants in a given area, including inoculation of ears, and c_1 is the additional cost associated with organizing and handling separate plots in the given area, including the cost of one laboratory determination per plot. If fixed costs are twice the additional costs, $c_1/c_2 = 0.5$, the optimum plot size is $e = 1.0$ ear per plot. If the additional cost, primarily assay determination costs, are eight times the fixed cost, $c_1/c_2 = 8$, the optimum plot size is $e = 4.0$ ears per plot. Whereas relative costs are difficult to define, this optimization indicates that small plots, say $e < 5$, be used unless the laboratory determinations are very expensive. The desired precision then would depend mainly on using the appropriate number of replicates.

Guidance for the number of replicates is provided by computing LSR values from LSDs. If $e = 4$, $d = 1$, and Student's $t = 2$, the LSR would be the antilog of the LSD calculated from $2\sqrt{[2(0.216)/r]}$. For replicate numbers of 10, 20, 40, 80, and 160, the LSR values are 2.60, 1.97, 1.61, 1.40, and 1.27, respectively, ie, for 10 replicates, a mean value would have to be more than 2.60 times larger than another mean value for a significant difference, or for 40 replicates, one mean would have to be 1.61 times larger than another. These calculations were made using estimates from experiment 4.

Although pinboard inoculation is a demanding activity that is expensive in time and effort, these estimates indicate that a large number of ears must be inoculated if stable estimates are to be obtained.

Experiments 1–3 gave estimates of experimental error that were smaller than that in experiment 4. These differences are probably due to differences of location or environment. Assay errors are probably not important, as indicated by the small size of that component in experiment 4. However, experiment 4 had a major difference in that only infected kernels were assayed, whereas the assays for the others were a blend of all kernels, of which probably fewer than 20% were infected. Another difference was that experiment 1 was conducted in controlled-environment rooms and the estimated variance components, except for $\hat{o}_d^2$, would not be expected to be similar to those in the field experiments. Differences in ear size among the crosses probably had little influence on mean aflatoxin levels. Crosses with the highest levels of aflatoxin also had about 25% greater grain yield.

These results indicate that an increase in precision can be expected by increasing the number of ears per plot and by increasing the number of replicates. The numbers of ears per plot were 1, 5, 5, and 4 and the numbers of replicates were 4, 2, 8, and 8 for experiments 1, 2, 3, and 4, respectively. From the components of variance considerations, it can be concluded that one should use small plots, ie, no more than five ears per plot, with a corresponding larger number of replicates. The calculations presented relate to the number of harvested ears per plot. Most plans include excess plants to ensure the desired final number and conform to the convenience of standardized plot size.

Gardner (3) examined the desired number of replicates for estimating aflatoxin by calculating coefficients of variation from single-ear data in an eight-parent diallel of 20 replicates. These calculations showed that using more than 10–12 replicates decreased the C.V.'s only slightly.

The experiences of the third author indicate that an effective testing program would be as follows: artificial inoculation—2 yr, 2 locations, 8 replicates, and 10 ears per plot with the inoculated kernels bulked; and natural infection—2 yr, 6–10 locations, 4 replicates, and all kernels from all ears of a plot bulked.

The data presented in this study indicate that resistance to accumulation of aflatoxin in developing corn kernels was under genetic control, that differences between duplicate aflatoxin assays were small, that variability among ears per plot was large, and that the greatest contribution to stable and economical estimates would come by increasing the number of replicates and keeping the number of ears to a low level of about five ears per plot.

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