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Article

Aflatoxin Accumulation in a Maize Diallel Cross

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Abstract: Aflatoxins, produced by the fungus *Aspergillus flavus*, occur naturally in maize. Contamination of maize grain with aflatoxin is a major food and feed safety problem and greatly reduces the value of the grain. Plant resistance is generally considered a highly desirable approach to reduction or elimination of aflatoxin in maize grain. In this investigation, a diallel cross was produced by crossing 10 inbred lines with varying degrees of resistance to aflatoxin accumulation in all possible combinations. Three lines that previously developed and released as sources of resistance to aflatoxin accumulation were included as parents. The 10 parental inbred lines and the 45 single crosses making up the diallel cross were evaluated for aflatoxin accumulation in field tests conducted in 2013 and 2014. Plants were inoculated with an *A. flavus* spore suspension seven days after silk emergence. Ears were harvested approximately 60 days later and concentration of aflatoxin in the grain determined. Parental inbred lines Mp717, Mp313E, and Mp719 exhibited low levels (3–12 ng/g) of aflatoxin accumulation. In the diallel analysis, both general and specific combining ability were significant sources of variation in the inheritance of resistance to aflatoxin accumulation. General combining ability effects for reduced aflatoxin accumulation were greatest for Mp494, Mp719, and Mp717. These lines should be especially useful in breeding for resistance to aflatoxin accumulation. Breeding strategies, such as reciprocal recurrent selection, would be appropriate.

Keywords: *Aspergillus flavus*; aflatoxin; maize; plant resistance

1. Introduction

Aflatoxins, produced by the fungus *Aspergillus flavus*, occur naturally in maize. The most potent carcinogens found in nature, aflatoxins are toxic not only to humans, but also to livestock, pets, and wildlife [1–4]. Dietary exposure to aflatoxins is one of the major causes of hepatocellular carcinoma, the fifth most common cancer in humans worldwide [5]. Their acute and chronic toxicity poses a major threat to humans in developing countries where maize is a dietary staple. The US Food and Drug Administration restricts the sale of grain with aflatoxin levels exceeding 20 ng/g [6]. In the United States aflatoxin contamination, first recognized as a major problem associated with maize production in the Southeast in the 1970s [7,8], continues to be a chronic problem in the Southeast and a sporadic problem in the Midwest [9,10]. Drought, high temperatures, and insect damage are often associated with aflatoxin contamination [8,9,11].

Plant resistance is generally considered a highly desirable approach toward reduction or elimination of aflatoxin accumulation in maize grain. Awareness of the serious consequences associated with pre-harvest aflatoxin contamination and the potential value of plant resistance in reducing losses to aflatoxin led to the establishment of several federally and state supported maize breeding programs in the southeastern USA to address the problem [12]. One such program was initiated by the U.S. Department of Agriculture's Agricultural Research Service at Mississippi State, Mississippi. The first line released from this program for resistance to *A.flavus* infection and aflatoxin accumulation was Mp313E [13]. Five additional lines were released subsequently: Mp420, Mp715, Mp717, Mp718, and Mp719 [14–17]. In addition, other potential useful sources of resistance have been identified [12,18].

This investigation was undertaken to compare aflatoxin accumulation in 10 maize inbred lines with varying levels of resistance to aflatoxin accumulation and in crosses among the 10 lines. The second objective was to determine the importance of general combining ability (GCA) and specific combining ability (SCA) in the inheritance to resistance to aflatoxin accumulation for this set of crosses.

2. Results

Among the 10 parental inbred lines, mean aflatoxin accumulation over years ranged from 742 ng/g for PHW79 to a low of 3 ng/g for Mp717 (Table 1). Aflatoxin concentration was lowest in Mp717, Mp313E, and Mp719, three lines that were developed and released as sources of resistance to *A. flavus* infection and aflatoxin accumulation by USDA-ARS [13,16,17]. NC388 also accumulated a low level of aflatoxin and did not differ significantly from Mp717 in mean aflatoxin concentration.

Differences in aflatoxin accumulation among single cross hybrids ranged from a high of 525 ng/g for Va35 × NC388 to a low of 2 ng/g for Mp313E × Mp494 and Mp313E × NC388 (Table 2). Interestingly, NC388 was a parent of the hybrids at both the high and low limits of aflatoxin accumulation. Mean aflatoxin accumulation was also high in B73 × T173 and Va35 × T173, crosses between two known susceptible lines. In addition to Mp313E × NC388 and Mp313E × Mp494, other hybrids that exhibited very low levels of aflatoxin accumulation (2–4 ng/g) were Mp719 × CML322, Mp719 × NC388, PHW79 × NC388, Mp717 × CML222, Mp313E × CML322, and Mp313E × Mp719.

Table 1. Mean aflatoxin accumulation in 10 parental inbred lines grown at Mississippi State in 2013 and 2014.

Inbred Line	Aflatoxin †	
	Logarithmic Mean [ln (y + 1)] ng/g	Geometric Mean ‡ ng/g
PHW79	6.61	742
B73	6.24	513
T173	5.96	387
Va35	5.04	154
CML322	5.04	154
Mp494	4.29	72
NC388	3.40	28
Mp719	2.60	12
Mp313E	2.49	11
Mp717	1.50	3
LSD(0.05)	2.28	

† Data were transformed (ln(y + 1), where y = concentration of aflatoxin in a sample) before statistical analysis; ‡ Geometric means were calculated by converting logarithmic means to the original scale.

Table 2. Mean aflatoxin accumulation in 45 F1 hybrids constituting a diallel cross grown at Mississippi State in 2013 and 2014.

F_1 Hybrid	Aflatoxin †	
	Logarithmic mean [ln (y + 1)] ng/g	Geometric Mean ‡ ng/g
Va35 × NC 388	6.27	525
B73 × T173	5.79	327
Va35 × T173	5.33	206
B73 × CML322	5.31	201
B73 × PHW79	5.07	158
PHW79 × Mp313E	4.83	125
Va35 × CML322	4.78	118
T173 × Mp719	4.76	116
T173 × CML322	4.69	108
B73 × Va35	4.48	87
Mp494 × CML322	4.41	81
Mp494 × Mp719	4.20	66
Mp717 × Mp719	4.00	54
T173 × Mp717	3.93	50
PHW79 × Mp494	3.81	44
Va35 × Mp494	3.76	42
B73 × Mp717	3.71	40
B73 × Mp313E	3.51	32
B73 × Mp719	3.45	31
B73 × Mp494	3.33	27
T173 × NC388	3.28	25
Mp494 × NC388	3.21	24

Table 2. *Cont.*

F_1 Hybrid	Aflatoxin †	
	Logarithmic mean [ln (y + 1)] ng/g	Geometric Mean ‡ ng/g
B73 × NC388	3.16	23
T173 × PHW79	3.18	23
Va35 × PHW79	3.13	22
T173 × Mp313E	3.00	19
Mp717 × NC388	2.63	13
Va35 × Mp717	2.46	11
NC388 × CML322	2.38	10
Va35 × Mp719	2.31	9
Mp313E × Mp717	22.5	9
Mp494 × Mp717	2.32	9
Va35 × Mp313E	2.22	8
T173 × Mp494	2.07	7
PHW79 × Mp717	1.79	5
PHW79 × Mp719	1.75	5
PHW79 × CML322	1.85	5
Mp313E × Mp719	1.54	4
Mp313E × CML322	1.69	4
Mp717 × CML322	1.53	4
PHW79 × NC388	1.36	3
Mp719 × NC388	1.30	3
Mp719 × CML322	1.30	3
Mp313E × Mp494	0.92	2
Mp313E × NC388	0.94	2
LSD (0.05)	2.02	

† Data were transformed (ln(y + 1), where y = concentration of aflatoxin in a sample) before statistical analysis; ‡ Geometric means were calculated by converting logarithmic means to the original scale.

The analysis of variance indicated that variation associated with years was not significant ($P < 0.05$) although the interaction of hybrids × years was a highly significant ($P < 0.01$) source of variation (Table 3). GCA was a highly significant ($P < 0.01$) source of variation in both 2013 and 2014 and in the combined analysis over years. SCA was a significant ($P < 0.01$) source of variation only in 2013. The interaction of GCA × years was not significant. Although SCA was not significant in the combined analysis, the interaction SCA × years was highly significant ($P < 0.01$).

GCA effects were calculated for 2013, 2014, and for the two years combined (Table 4). GCA effects were highly significant ($P < 0.01$) and negative for Mp494, Mp717, Mp719, and NC388 each year and over years. In an earlier investigation of a diallel cross produced from a different set of lines, Mp717 and Mp494 exhibited highly significant GCA effects for reduced aflatoxin accumulation [18]. GCA effects for aflatoxin accumulation were highly significant ($P < 0.01$) and positive for PHW79 in 2013, 2014, and over years. GCA effects for T173 and B73 were significant ($P < 0.05$) and positive in the combined analysis, but highly significant ($P < 0.01$) within each year. PHW79, B73, and T173 contributed to increased susceptibility when used in production of hybrids.

Table 3. Analysis of variance for aflatoxin concentration in grain harvested from a diallel cross grown in 2013 and 2014 at Mississippi State.

Source	df	Mean squares [ln(y+1)] ng/g †		
		2013	2014	Over Years
Years	1	-	-	0.67
Reps (year)	6	-	-	1.18
Hybrids	44	12.24 **	7.19 **	15.50 **
GCA ‡	9	43.23 **	25.27 **	65.59 **
SCA ‡	35	4.21 **	2.61	2.61
Hybrids × Years	44	-	-	3.98 **
GCA × Years	9	-	-	3.01
SCA × Years	35	-	-	4.23 **
Error	132,264 §	1.88	1.73	1.81

** Significant at $P < 0.01$; † Data were transformed (ln(y + 1), where y = concentration of aflatoxin in a sample) before statistical analysis; ‡ GCA, general combining ability; SCA = specific combining ability; § df = 132 for individual years and 264 for combined years.

Table 4. Estimates of general combining ability (GCA) effects for 10 inbred parental lines of a diallel cross of maize grown at Mississippi State in 2013 and 2014.

Inbred Line	GCA effects [ln(y + 1)] ng/g †		
	2013	2014	Over Years
PHW79	2.10 **	1.26 **	1.68 **
T173	1.46 **	1.07 **	1.27 *
B73	1.16 **	0.87 **	1.01 *
Va35	0.09	0.81 **	0.45
Mp313E	−0.39	−0.37	−0.38 *
CML322	−0.60 **	−0.31	−0.46 **
NC388	−0.68 **	−0.72 **	−0.70 **
Mp717	−0.84 **	−0.87 **	−0.85 **
Mp719	−1.13 **	−0.77 **	−0.95 **
Mp494	−1.17 **	−0.96 **	−1.07 **

* Significantly different from 0 at $P < 0.05$; ** Significantly different from 0 at $P < 0.05$; † Data were transformed (ln(y + 1), where y = concentration of aflatoxin in a sample) before analysis.

3. Discussion

Aflatoxin accumulation in the inbred lines per se (Table 1) was generally consistent with their performance in hybrid combinations as indicated by the GCA effects (Table 4). The susceptible lines, PHW79, B73, T173, and Va35, had the greatest accumulation of aflatoxin as inbred lines and the highest positive GCA effects. Although aflatoxin accumulation was only 11 ng/g for Mp313E, the second lowest among the inbred lines, the associated GCA effect for Mp313E indicated the least reduction in aflatoxin accumulation among the resistant lines. This is consistent with the results of a previous investigation in which Mp313E was included in a diallel cross produced from a different set of lines [18]. The negative GCA effects associated with Mp494, Mp717, and Mp719, lines that were developed specifically as sources of resistance to accumulation of aflatoxin, indicate that these lines

could be useful in breeding programs. Although NC388 was not developed and released as a source of resistance, it appears to have potential for use in breeding for resistance as well. Breeding methods that maximize the use of GCA should be most effective in using these lines to develop germplasm with resistance to aflatoxin accumulation.

Although conventional breeding strategies based on phenotypic selection should be useful in the production of improved germplasm lines and hybrids, molecular marker-assisted selection should be effective in increasing resistance to aflatoxin accumulation as well. USDA-ARS at Mississippi State has invested considerable effort in mapping quantitative trait loci (QTL) associated with resistance to aflatoxin accumulation [19] and using that information to develop near-isogenic lines. Mp313E was the primary source of resistance in much of this effort. Mp494, Mp717, Mp719, and NC388, the lines that exhibited highly significant GCA effects for resistance in this investigation, may prove to be a better source of QTL, and eventually gene-based markers, for resistance to aflatoxin accumulation. For a trait such as resistance to aflatoxin accumulation that is highly sensitive to environmental effects and variations, molecular markers may well be the most effective approach to efficiently producing resistant hybrids. Investigations such as this one can be useful in choosing the best sources of germplasm for inclusion in gene-mapping studies. Efforts to identify gene-based molecular markers for resistance in Mp494, Mp719, and other resistant lines are currently underway.

4. Materials and Methods

A diallel cross was produced by crossing 10 inbred lines of maize in all possible combinations. Three of the parental inbred lines were developed and released by USDA-ARS at Mississippi State as sources of resistance to *A. flavus* infection and aflatoxin accumulation: Mp313E, Mp717, and Mp719 [13,16,17]. Mp494, NC388, and CML322 had exhibited resistance, and Va35, T173, B73, and PHW79, susceptibility in field trials conducted at Mississippi State [12,18,20]. The 10 inbred lines were planted on 14 May 2013 and 21 April 2014 in a Leeper silty clay loam (fine, smectitic, non-acid, thermic Vertic Epiaquepts) soil at Mississippi State, MS. In separate experiments, the 45 hybrids constituting the diallel cross were planted on 15 May 2013 and 21 April 2014. Both inbred lines and hybrids were planted in single row plots that were 4 m long, spaced 0.97 m apart, and arranged in a randomized complete block design with four replications. Standard maize production practices for the area were followed.

Seven days after silks had emerged from 50% of the plants in a plot, the primary ear of each plant was inoculated with *A. flavus* isolate NRRL 3357, which is known to produce aflatoxin in maize, using the side-needle technique [21]. Using a tree-marking gun fitted with a 14-gage needle, a 3.4 mL suspension containing 3×10^8 *A. flavus* conidia was injected underneath the husks into the side of the ear. Inoculum was prepared as described by [22].

The inoculated ears were harvested approximately 60 d after inoculation and dried at 38° for 7 day. Ears from each plot were bulked and shelled. The grain was thoroughly mixed before grinding with a Romer mill (Union, MO, USA). The concentration of aflatoxin in a 50-g sample was determined by the Vicam AflaTest procedure (Watertown, MA, USA). This procedure detects aflatoxin at levels as low a 1 ng/g.

The values for aflatoxin concentration were transformed before statistical analysis as (ln(y + 1), where y is the concentration of aflatoxin in a sample). The transformation was performed to provide a more nearly normal distribution of the data. Data were analyzed using the SAS General Linear Models procedure [23]. In the analysis of the diallel cross, the variance was partitioned using DIALLEL-SAS [24,25] based on Griffing's [26] Method 4, Model I, into GCA and SCA components and their interactions with years. To test the significance of GCA and SCA components in the 2-year analysis, the interactions between years and the corresponding component as the error term for *F*-tests. Estimates of GCA and SCA were calculated and their significance determined by *t*-tests.

5. Conclusions

The identification of maize germplasm with heritable resistance to *A. flavus* infection, and the subsequent accumulation, is important in reducing aflatoxin contamination of maize. A diallel cross, produced by crossing 10 parental lines with varying degrees of resistance, was evaluated for resistance to aflatoxin accumulation in field tests. Both GCA and SCA were significant sources of variation in the inheritance of resistance to aflatoxin accumulation. GCA effects for reduced aflatoxin accumulation were greatest for Mp494, Mp717, and Mp719 indicating that these lines should be especially useful in breeding for resistance to aflatoxin accumulation in maize. Conventional breeding strategies such as reciprocal recurrent selection should be effective in increasing levels of resistance. Mp494, Mp717, and Mp719 are also potentially useful sources of gene-based markers for resistance to aflatoxin accumulation.

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Author Contributions

W.W. conceived and designed the study, produced the seed, analyzed the data, and wrote the manuscript. G.W. inoculated developing ears with *A. flavus*, analyzed grain samples for aflatoxin content, and summarized the data.

Conflicts of Interest

The authors declare no conflicts of interest.

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