# Sources of Resistance to Aflatoxin Production in Maize

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Drought-tolerant maize genotypes (Huffman, Z08-004, Tuxpan, PH 9, NRC 5348, Chunco, Saint Croix, and Arizona) were compared in the field and laboratory to toxin-resistant GT-MAS:gk and Yellow Creole. SDS-PAGE, scanning electron microscopy of kernel cuticle, amount of kernel wax, *Aspergillus flavus* kernel colonization, Aspergillus ear rot, insect damage, aflatoxin production, and their relationships were examined. SDS-PAGE showed the presence of a 14 kDa trypsin inhibitor in the kernels of all genotypes except Chunco, which contains a protein of a larger molecular weight. The 14 kDa trypsin inhibitor protein content in these genotypes was higher than in GT-MAS:gk and Yellow Creole. Scanning electron microscopy revealed that Arizona, Huffman, and Chunco genotypes had abundant wax deposits on kernel surfaces and the amount of pericarp wax was equal to or above that from GT-MAS:gk and Yellow Creole. Differences in Aspergillus ear rot ratings, fungal colonization, and insect damage by corn earworm were observed in all drought-tolerant maize genotypes as well as in the controls. Kernel screening assays showed that aflatoxin $B_1$ levels in inoculated drought-tolerant genotypes differed significantly from those in GT-MAS:gk and Yellow Creole (LSD = 576). Aflatoxin $B_1$ levels in the inoculated genotypes differed significantly from those of GT-MAS:gk or Yellow Creole (LSD = 1389) when grown under drought stress conditions. Pearson correlation coefficients were significant between ear rot ratings and insect damage ($r = 0.75$; $P = 0.01$) and between Aspergillus ear rot and aflatoxin levels ($r = 0.54$; $P = 0.05$). On the basis of the parameters studied, there are indications that these genotypes were potential sources of *A. flavus* resistance.

**Keywords:** *Food safety; mycotoxin; protein; scanning electron microscopy; Zea mays*

## INTRODUCTION

Contamination of food and feed grains by aflatoxins is a problem throughout the world. Aflatoxins, which are toxic secondary metabolites produced by *Aspergillus flavus* Link ex Fries and *Aspergillus parasiticus* Speare, are potent carcinogens to animals and have been linked to liver cancer in humans (*1*−*3*).

Substantial effort has been made to identify corn genotypes that resist infection by *A. flavus* (*4*−*6*). Although the most effective control of *A. flavus* and aflatoxin contamination is through the development of genetically resistant hybrids (*7*), successful management of aflatoxin in the field will require host resistance combined with management strategies such as appropriate nitrogen fertilization, population densities, insect control, and irrigation (*8*−*12*). Widstrom et al. (*13*) reported that the corn population GT-MAS:gk consistently showed resistance when compared with susceptible hybrids. In repeated tests, fungal colonization in individual inoculated kernels of GT-MAS:gk was reduced, and aflatoxin levels in this genotype were lower than those in susceptible hybrids (*5*). High-temperature and drought conditions stress the plant and can result in high aflatoxin levels (*9*, *14*, *15*). Plant stress facilitates greater colonization of corn kernel and infection by *A. flavus* in the field (*9*, *14*). Colonization of the kernel by this fungus plays an important role in the epidemiology of this disease (*16*, *17*). Damaged kernel pericarps are usually the result of insect feeding, and previous researchers have associated insect damage to ears with increased fungal sporulation and aflatoxin production (*18*). Guo et al. (*5*) showed that resistance is associated with a preformed compound or compound within the kernel, as well as wax and cutin in the intact kernel pericarp. Previous reports by Eigenbrode et al. (*19*), Espelie et al. (*20*), and Bergman et al. (*21*) showed that variations in chemical and ultrastructural characteristics of the plant surface may affect many aspects of insect behavior such as oviposition, orientation, and feeding and thereby result in differential host-plant resistance. A more complete identification and characterization of proteins would allow for more efficient incorporation of these characteristics into breeding programs.

There has been little or no emphasis on breeding for drought tolerance to limit aflatoxin production. Selection for this trait has been difficult because its expression in the field cannot be generated at will (*2*). It is possible that various germplasm collections may contain resistance to *A. flavus* and aflatoxin production that have remained undetected. The objective of this study was to compare known drought-tolerant maize genotypes to toxin-resistant GT-MAS:gk and Yellow Creole and to identify those that might be used as sources of resistance to Aspergillus ear rot and aflatoxin production.

## MATERIALS AND METHODS

**Laboratory Studies. Protein Extraction and Gel Electrophoresis.** Dry kernels (15 g) of each genotype (Huffman, Z08-004, Tuxpan, PH 9, NRC 5348, Chunco, Saint Croix, Arizona, Gt-MAS:gk, and Yellow Creole) were extracted for protein as described by Chen et al. (*22*). Sodium dodecyl

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sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) of protein extracts was performed using a 15% resolving gel with a 4% stacking gel. Sigma low-range protein markers (Sigma Chemical Co., St. Louis) were used as molecular mass standards. The gels were electrophoresed (120 V, 1.5 h), stained with 0.125% Coomassie blue R-250 in 50% methanol and 10% acetic acid for 1 h at room temperature, and destained in 50% methanol and 10% acetic acid. The quantitation of the 14 kDa protein (percent protein content) was performed as previously described by Chen et al. (*22*). The genotypes were replicated three times and repeated.

**Scanning Electron Microscopy of Kernel Wax.** Genotypes Huffman, Z08-004, Tuxpan, PH 9, NRC 5348, Chunco, Saint Croix, and Arizona and two controls, GT-MAS:gk and Yellow Creole, were supplied by USDA Plant Introduction in Ames, IA. Ten kernels of each genotype were prepared and examined for kernel wax appearance as previously described (*6*).

**Amount of Kernel Wax.** Weight of wax from Huffman, Z08-004, Tuxpan, PH 9, NRC 5348, Chunco, Saint Croix, Arizona, and two controls, GT-MAS:gk and Yellow Creole, was determined according toby the procedure employed in an earlier study (*6*). In tared flasks, 40 kernels of each genotype were immersed in 100 mL of chloroform for 60 s. Care was taken to clean the kernels of all debris prior to immersion. The kernels were removed, the chloroform was evaporated to dryness, and the weight of the wax from each genotype was determined. The genotypes were replicated five times and repeated.

**Kernel Screening Assay (KSA).** Kernels of 10 maize genotypes were surface-sterilized, dipped into a suspension of *A. flavus* strain AF13 conidia ($10^6$ conidia/mL), and evaluated using the KSA (*4*). Strain AF13, which produces abundant aflatoxin $B_1$ (*23*), was used in laboratory studies. The fungus was grown on V-8 juice agar (5% V-8 juice, 2% agar) for 10 days at 28 °C in darkness. The concentration of dislodged conidia was determined using a hemocytometer and diluted to $10^6$ conidia/mL. Each experimental unit contained four kernels that were replicated six times. A fungal colonization rating of 1−5 (1 = 1−20% and 5 = 81−100% of the kernel surface covered by conidiophores bearing conidia) was determined for each replicate and averaged for each genotype. Kernels were incubated at 30 °C for 7 days, after which time they were removed, dried in a forced-air oven at 60 °C for 3 days, and prepared for aflatoxin analysis. The experiment was performed twice.

**Field Studies. General Procedure.** Field experiments were conducted at Baton Rouge and Winnsboro, LA. The soil was prone to drought (*12*). All plants received only 50 kg/ha N. Ten maize genotypes were inoculated using the pinbar technique (*24*). Maize genotypes at these locations were planted in two-row plots at ∼38500 seeds/ha. Experimental units consisted of a 3-m-long section of row in each plot. Plots were arranged in a randomized complete block design with four replications. Atrazine [1.5 lb of active ingredient (ai)/acre], alachlor (1.0 lb of ai/acre), and 0.5% surfactant were applied preemergence for weed control. The insecticide, Terbufos (1.0 lb of ai/acre), was applied in furrow at planting.

Strain AF13 was also used in all field studies. The fungus was grown on V-8 juice agar (5% V-8 juice, 2% agar) for 10 days at 28 °C in darkness as described above. Approximately 20 days after silk emergence, the upper ear was inoculated with AF13 conidial suspension using the pinbar technique (*24*). Ten ears per plot were harvested by hand when kernels reached ∼15% moisture, dried, and assessed for corn earworm damage (CEW) caused by *Helicoverpa zea* Boddie. Insect damage was assessed by measuring the depth of penetration (centimeters) from the tip to the base of the ear (*18*). A visual Aspergillus ear rot rating of 1−10 (1 = 10% and 10 = 100% of inoculated area rotted) was determined for each ear and averaged for each plot (*25*).

Ears were machine shelled, and kernel moisture content was determined. Shelled grain was bulked and dried at 60 °C to ∼13% moisture in a forced-air dryer. Subsamples (500 g) from each plot were ground in a Wiley mill (model 4) to a particle size of 1 mm and stored at 4 °C until used for aflatoxin extraction. Treatments were replicated four times (50 g of ground corn/replicate).

**Table 1. Quantitation of the Relative Content of the 14 kDa Trypsin Inhibitor Protein Band in the Total Kernel Protein Extract of the 10 Drought-Tolerant Maize Genotypes Based upon Analysis of 15% SDS-PAGE Profiles after Staining with Coomassie Brilliant R-250**

| genotype[a] | accession | source country | 14 kDa trypsin inhibitor protein content (%) |
|---|---|---|---|
| Huffman | Ames 3124 | United States (TN) | 17.89 |
| Yellow Creole[b] | Ames 3125 | United States (LA) | 11.86 |
| GT-MAS:gk[b] | PI 561859 | United States (GA) | 10.12 |
| PH 9 | PI 391414 | Philippines | 13.50[c] |
| Tuxpan | PI 483314 | United States (SC) | 17.45 |
| Z008−004 | PI 474210 | Mexico, Sonora | 15.43 |
| Chunco | PI 390840 | Peru | —[d] |
| NRC 5348 | PI 218180 | United States (AZ) | 13.78[e] |
| Arizona | PI 508270 | United States (AA) | 18.12[c] |
| St. Croix | PI 504146 | Virgin Islands (U.S.) | 19.21[e] |

[a] Seed provided by the USDA Plant Introduction, Ames, IA. [b] Controls. [c] Contains a 34 kDa Pro-RIP protein. [d] The 14 kDa is absent. [e] Contains a 23 kDa protein.

**Aflatoxin Extraction and Thin-Layer Chromatography (TLC).** Levels of aflatoxin $B_1$ were determined using official methods of the American Oil Chemists' Society (*26*). The ground corn (50 g) was combined with 100 mL of methylene chloride in a 250-mL flask and placed for 30 min in a rotary shaker at 200 rpm. The contents of the flask were filtered through Whatman No. 1 paper, and solvent was allowed to evaporate to dryness under a fume hood. The residues were dissolved in 2 mL of benzene/acetonitrile (98:2), spotted (10 $\mu$L) on silica gel TLC plates (EM Science, Gibbstown, NJ), and developed in ether/methanol/water (96:3:1). Aflatoxin $B_1$ was quantified using a scanning densitometer with a fluorometry attachment (model CS-930; Shimadzu Scientific Instruments, Inc., Tokyo, Japan). A commercial aflatoxin B and G mixture (Sigma) served as a standard. Criteria for purity of aflatoxin primary standards and determination of concentration of diluted working standards for TLC plates have been described (*26*). The scanning densitometer with a fluorometry attachment can detect aflatoxins ($B_1$, $B_2$, $G_1$, and $G_2$) at concentrations as low as 1 ng/g (*10*). The same procedure was used for KSA.

**Statistical Analysis.** Data from insect damage, ear rot, amount of kernel wax, fungal colonization, and ($AFB_1$) values for each environment were analyzed separately and combined using the GLM procedure of SAS (SAS Institute, Cary, NC). Means were separated using the least significant difference (LSD) ($P \leq 0.05$). Prior to analysis, data on $AFB_1$ values were transformed using $\log(X + 1)$. Pearson correlation coefficients also were calculated between Aspergillus ear rot, insect damage, amount of wax, fungal colonization, and aflatoxin $B_1$ values.

## RESULTS AND DISCUSSION

SDS-PAGE showed the presence of a 14 kDa trypsin inhibitor protein in kernels of all genotypes except Chunco, which contained a protein of a larger molecular weight. This 14 kDa protein content in drought-tolerant maize genotypes was higher than in controls GT-MAS:gk and Yellow Creole (Table 1). Among drought-tolerant genotypes, NRC 5348 and St. Croix appear to contain a 23 kDa protein and genotypes PH 9 and Arizona contain a 34 kDa Pro-RIP protein (Table 1).

The resistance to *A. flavus* infection and subsequent aflatoxin production observed in some of these genotypes may be due in part to this 14 kDa trypsin inhibitor in kernels reported earlier by Chen et al. (*22*). The mode of action of this 14 kDa protein against fungal growth has been suggested to be in part because of its inhibition

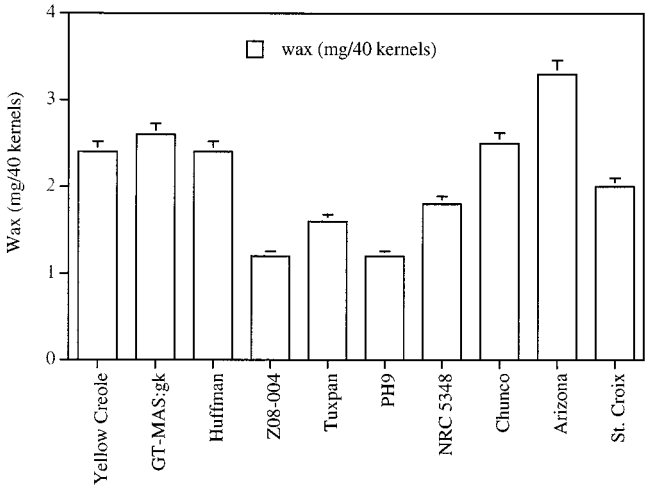


**Figure 1.** Pericarp wax from kernels of drought-tolerant maize genotypes and controls GT-MAS:gk and Yellow Creole. Wax was removed from kernels provided as seed from USDA Plant Introduction, Ames, IA.

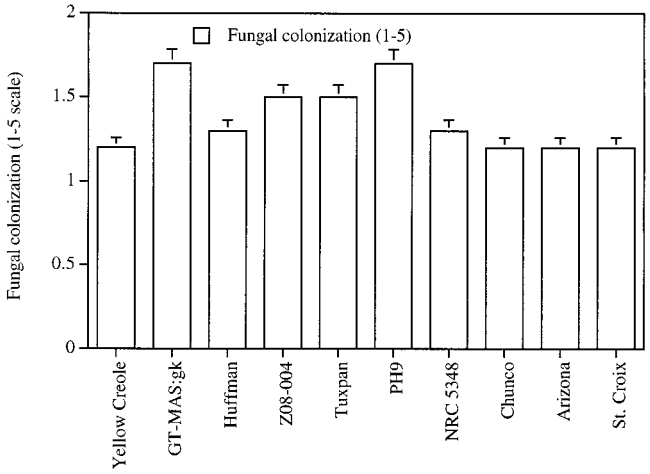


**Figure 2.** Colonization by *A. flavus* on kernels of drought-tolerant maize genotypes and controls GT-MAS:gk and Yellow Creole in a kernel screening assay. Fungal colonization ratings were on a 1−5 scale (1 = 1−20% and 5 = 81−100% of kernel surface covered by conidiophores bearing conidia). Seeds were provided by USDA Plant Introduction, Ames, IA.

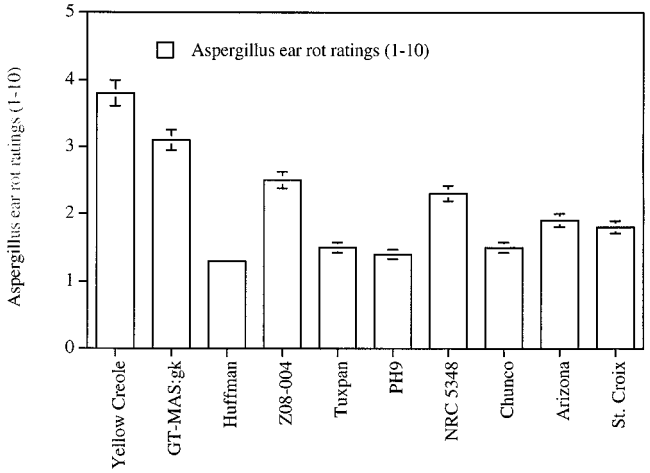


**Figure 3.** Aspergillus ear rot ratings on kernels of drought-tolerant maize genotypes and controls GT-MAS:gk and Yellow Creole. Ear rot ratings were on a 1−10 scale (1 = 10% and 10 = 100% of inoculated area rotted). Seeds were provided by USDA Plant Introduction, Ames, IA.

of fungal α-amylase, limiting *A. flavus* access to simple sugars (*27*). The presence of this protein at high concentration in all drought-tolerant genotypes and the reduction of aflatoxin in most of these genotypes suggest that this protein may play a role as earlier reported by Chen et al. (*22*) and Hojima et al. (*28*).

Scanning electron microscopy (SEM) revealed distinct differences among maize genotypes. Kernels of maize genotypes Arizona, Chunco, and Huffman were rough in appearance and had abundant wax deposits on kernel surfaces similar to those of GT-MAS:gk and Yellow Creole. Kernel wax of Z08-004, PH 9, and Tuxpan appeared to be smoother and lacked the abundant surface deposits observed in other genotypes. The difference between kernel wax of drought-tolerant genotypes found by SEM was supported by the results obtained by the weight of kernel wax. In an earlier study (*6*), using SEM, GT-MAS:gk appeared to be rough and showed abundant wax deposits on kernel surfaces, whereas susceptible kernels appeared to be smoother and lacked the abundant surface deposits observed in GT-MAS:gk.

No significant test by treatment interaction for kernel wax weight was found. Therefore, data from duplicate tests were combined for final analysis. Kernels of genotypes Arizona had 27−38% more wax than GT-MAS:gk and Yellow Creole, respectively (Figure 1). Genotypes Z08-004, Tuxpan, and PH 9 had significantly less pericarp wax than those of control GT-MAS:gk and Yellow Creole. Amounts of kernel wax of GT-MAS:gk, Yellow Creole, Huffman, and Chunco did not differ from one another. We also showed that kernels of GT-MAS:gk had more surface wax than did susceptible commercial hybrids tested. This suggested that resistance to *A. flavus* in GT-MAS:gk may be caused in part by a physical barrier provided by pericarp wax. The pericarp is the outermost portion of corn kernels and is composed of several layers of cells differing in chemical components and cell wall thickness (*29*). The pericarp contains layers of cutin and wax, which afford considerable protection against invasion by fungal pathogens (*30, 31*). Several researchers have shown that pericarp is important for protecting seeds of certain crop species from *A. flavus* infection and aflatoxin production (*32, 33*). They suggested a role of seed surface in this resistance. Because of the presence of abundant surface wax deposits on kernels of Chunco, Arizona, and Huffman genotypes, as well as more surface wax, the study confirms that seed surface wax may play a role in kernel resistance to aflatoxin production. The difference observed on kernel wax appearance may be the result of a difference in chemical composition of these kernel waxes and other unknown factors. Antifungal activity against *A. flavus* in cuticular lipids from certain insect has been demonstrated by Koidsumi (*34*). The active constituents were free medium-chain-length saturated fatty acids, such as caprylic acid or capric acid.

PH 9, Z08-004, Tuxpan, and GT-MAS:gk had significantly higher *A. flavus* colonization ratings than the other six genotypes (Figure 2); however, colonization of kernels of GT-MAS:gk by *A. flavus* did not differ from that in PH 9. Genotypes differed significantly for Aspergillus ear rot ratings. Ear rot ratings ranged from 1.3 in Huffman to 3.8 in control Yellow Creole (Figure 3). Environmental conditions during the growing seasons were favorable for Aspergillus ear rot because of higher than average temperature and lower than average rainfall.

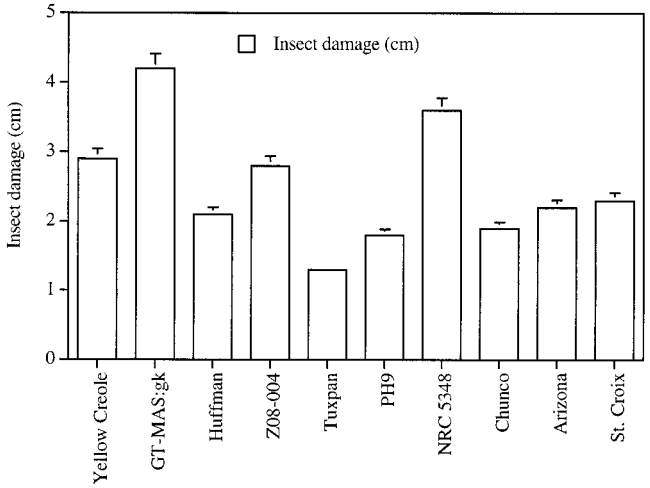


**Figure 4.** Insect damage (cm) by corn earworm (*H. zea* Boddie) from drought-tolerant maize genotypes and controls GT-MAS:gk and Yellow Creole. Seeds were provided by USDA Plant Introduction, Ames, IA.

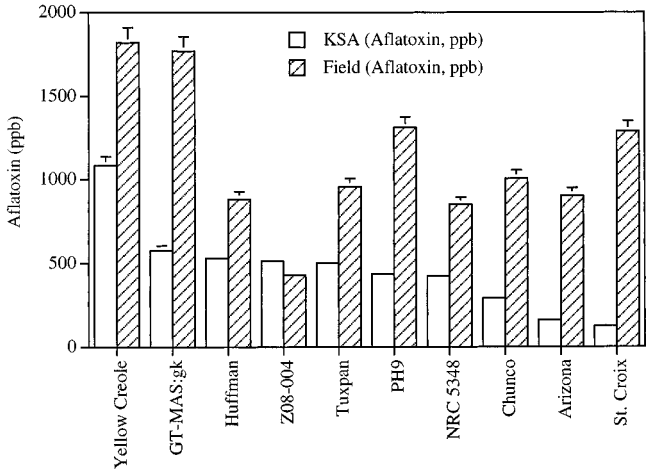


**Figure 5.** Aflatoxin $B_1$ (parts per billion) levels from drought-tolerant maize genotypes and controls GT-MAS:gk and Yellow Creole (field and laboratory). Seeds were provided by USDA Plant Introduction, Ames, IA.

Genotypes differed significantly for insect damage. Insect damage ranged from 1.3 cm in Tuxpan to 4.2 cm in control GT-MAS:gk (Figure 4). GT-MAS:gk and NRS 5348 sustain more insect damage than Huffman, Z08-004, Arizona, and St. Croix, whereas Tuxpan, PH 9, and Chunco were less damaged by corn earworm (CEW). Genotypes resistant to insect damage did not always have lower aflatoxin values and fungal colonization. Reports by Yang et al. (*35*, *36*) showed that in some instances the cuticular lipids inhibited fall armyworm (FAW) (*Spodoptera frugiperda* J.E. Smith) development, whereas total lipid extract did not inhibit fall armyworm growth. Because total lipid extract did not inhibit fall armyworm growth and because individual surface lipid component enhanced larval growth, it can be concluded that the role of the cuticular lipids of corn in FAW feeding behavior is unclear.

No significant test by treatment interaction for $AFB_1$ production was found. Therefore, data from duplicate tests were combined for final analysis. KSA showed that aflatoxin $B_1$ levels in inoculated drought-tolerant genotypes differed significantly from those in GT-MAS:gk and Yellow Creole (LSD = 576). Aflatoxin $B_1$ levels in the inoculated genotypes also differed from those of GT-MAS:gk or Yellow Creole (LSD = 1389) when the corn was grown under drought stress conditions (Figure 5). This suggests the possible association of drought tolerance and aflatoxin resistance. The soil was prone to drought because of its low water-holding capacity, its low water infiltration rate, and the unfavorable physical and chemical properties of the subsoil that reduce root depth substantially (*12*).

Pearson correlation coefficients were significant between Aspergillus ear rot rating and insect damage ($r = 0.75$; $P = 0.01$) and between Aspergillus ear rot rating and $AFB_1$ levels ($r = 0.54$; $P = 0.05$). Overall, genotypes with low Aspergillus ear rot ratings had lower $AFB_1$ levels. Pearson correlation coefficients were high ($r = 0.73$) between Aspergillus ear rot rating and insect damage and moderately high ($r = 0.54$) between Aspergillus ear rot rating and $AFB_1$ content. These results imply a relationship between *A. flavus* ear rot rating and insect damage and *A. flavus* ear rot rating and aflatoxin production. Our finding confirms previous results of Campbell and White (*25*) and Tucker et al. (*37*). They reported a moderately high Pearson coefficient of correlation ranging from $r = 0.52$ to $r = 0.80$ between Aspergillus ear rot ratings and $AFB_1$ content. Results from previous studies (*16*, *25*, *38*−*40*) together with ours showed no correlation between insect damage and aflatoxin production. The interaction between insects and *A. flavus* is complex and difficult to document. Lack of correlation between insect damage and aflatoxin observed in previous studies (*16*, *25*, *38*−*40*) has been attributed to the weather, insect population and variability, and genotype differences. Results show that various germplasm collections may contain resistance to *A. flavus* and aflatoxin production that have remained undetected as these genotypes used in the study demonstrate. These genotypes might be used as potential sources of resistance to Aspergillus ear rot and aflatoxin production.

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