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Research Article

Comparison of Two Inoculation Methods for Evaluating Maize for Resistance to *Aspergillus flavus* Infection and Aflatoxin Accumulation

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Aflatoxin, the most potent carcinogen found in nature, is produced by the fungus *Aspergillus flavus* and occurs naturally in maize, *Zea mays* L. Growing maize hybrids with genetic resistance to aflatoxin contamination are generally considered a highly desirable way to reduce losses to aflatoxin. Developing resistant hybrids requires reliable inoculation methods for screening maize germplasm for resistance to *A. flavus* infection and aflatoxin accumulation. The side-needle technique is a widely used inoculation technique: an *A. flavus* conidial suspension is injected underneath the husks into the side of the ear. This wounds the ear and limits expression of resistance associated with husk coverage, pericarp thickness, and seed coat integrity. In this investigation, the side-needle technique was compared with a second inoculation method that involved dispensing wheat kernels infected with *A. flavus* into plant whorls at 35 and 49 days after planting. Results showed that although the side-needle technique produced higher levels of aflatoxin accumulation, differences in *A. flavus* biomass produced by the two inoculation techniques were not significant. Both inoculation techniques were effective in differentiating resistant and susceptible single cross hybrids irrespective of the use of *A. flavus* infection or aflatoxin accumulation as a basis to define resistance.

1. Introduction

Aflatoxin is produced by the fungus *Aspergillus flavus* and occurs naturally in maize, *Zea mays* L. Aflatoxin, the most potent carcinogen found in nature, is toxic to both humans and animals [1–3]. Dietary exposure to aflatoxin is one of the major causes of hepatocellular carcinoma, the fifth most common cancer in humans worldwide [4]. Although aflatoxin was first identified and recognized as a threat to animals after 100,000 turkeys died in England in 1961 [5–7], it is now known to be a threat to other livestock, pets, and wildlife [8–10]. The U. S. Food and Drug Administration restricts the sale of grain with aflatoxin levels exceeding 20 ng/g [2]. Aflatoxin was first recognized as a major problem for maize production in the southeastern United States in the 1970s. In 1977, over 90% of maize was contaminated with aflatoxin, and aflatoxin levels exceeded 20 ng/g in 90% of samples evaluated in Georgia [11, 12]. Aflatoxin contamination has remained a chronic problem in the Southeast where it reached devastating proportions in 1998 [13, 14]. Losses to aflatoxin-contaminated corn in Arkansas, Louisiana, Mississippi, and Texas were estimated at $85,000,000 to $100,000,000 [15]. An increase in the use of maize as a substrate for ethanol production further exacerbates losses from aflatoxin contamination: concentration of aflatoxin in distillers' dry grain during fermentation represents a serious impediment to its use in animal feeds [16, 17]. Plant resistance is generally considered a highly desirable strategy for reducing or eliminating aflatoxin contamination, but commercial maize hybrids with adequate levels of resistance to aflatoxin contamination are not currently available [18, 19]. Identification and release of maize germplasm with high levels of resistance to *A. flavus* infection and aflatoxin

accumulation and associated molecular markers are essential to the efficient production of resistant maize hybrids. Identifying germplasm with resistance to a pest or pathogen requires a source of germplasm and reliable techniques for evaluating the germplasm.

The USDA-ARS Corn Host Plant Resistance Research Unit at Mississippi State, Mississippi, has been at the forefront in developing techniques for evaluating maize germplasm for resistance to *A. flavus* infection and aflatoxin accumulation. Because natural infection by *A. flavus* and subsequent aflatoxin production are sporadic from one growing season to the next, artificial inoculation techniques were developed to uniformly infect developing maize ears with the fungus [20]. *A. flavus* is considered to be a weak pathogen, and most of the early inoculation techniques focused on wounding kernel and cob tissue [21–26]. Methods such as the pinbar and pinboard [26] severely wounded ears and produced high levels of *A. flavus* infection and aflatoxin contamination in the hot, humid environment of the southeastern U.S. The side-needle technique has been used as the standard method for inoculating maize with *A. flavus* for the past 20 years [20, 25]. This technique wounds few kernels but provides consistently high levels of infection in maize lines used as susceptible checks. Inoculation methods are needed that are less labor intensive, mimic natural infection, and are suitable for use in large scale field evaluations. Spray techniques have been used to inoculate field tests with *A. flavus* and appear to be most effective in drought prone soils [27, 28]. Applications of *A. flavus* infected materials have been used to inoculate maize in field studies [29].

In the current investigation, an inoculation method using *A. flavus* infected wheat, *Triticum aestivum*, kernels dispensed into the plant whorls was evaluated for screening maize germplasm for resistance to *A. flavus* infection and subsequent accumulation of aflatoxin. The effectiveness of this technique was compared with that of the side-needle technique for identifying maize germplasm with resistance to aflatoxin accumulation.

2. Materials and Methods

2.1. Plant Materials and Field Trials. Ten single cross maize hybrids were evaluated for resistance to *A. flavus* infection and subsequent accumulation of aflatoxin using two methods of inoculation with *A. flavus* in field trials conducted at Mississippi State, Mississippi, in 2011 and 2012. The single crosses were selected to represent a broad range of resistance and were generated by crossing maize lines with varying levels of resistance to aflatoxin accumulation. Germplasm lines Mp313E, Mp494, Mp715, Mp717, and Mp04 : 127 were developed at Mississippi State as sources of resistance and have been useful in developing hybrids with reduced aflatoxin accumulation [30–34]. TZAR101 was developed at the International Institute of Tropical Agriculture (IITA) in Nigeria as a source of resistance [35]. The other parental inbred lines are susceptible to aflatoxin accumulation.

The 10 single cross maize hybrids were planted on 10 May 2011 and on 11 April 2012 in a Leeper silty clay loam (fine, smectitic, nonacid, and thermic Vertic Epiaquepts) soil at the R. R. Foil Plant Science Research Center, Mississippi State University. The experimental design was a randomized complete block with four replications and a split plot treatment arrangement. Methods of inoculation with *A. flavus* were assigned to main plots; hybrids were assigned to subplots consisting of single rows that were 4 m long and spaced 0.90 m apart. The single-row plots were overplanted and thinned to 20 plants. Standard production practices for the area were followed.

2.2. Inoculation Procedures. Two methods of inoculating plants with *A. flavus* were compared. The side-needle technique was developed in the USDA-ARS Corn Host Plant Resistance Research Unit at Mississippi State University and has been the favored inoculation technique for many years. *A. flavus* isolate NRRL 3357, which is known to produce high levels of aflatoxin, was increased on sterile corn cob grits (size 2040, Grit-O-Cobs, Maumee, Ohio) in 500 m flasks, each containing 50 g of grits and 100 mL sterile distilled water, and incubated at 28°C for 21 days. Conidia in each flask were washed from the grits with 500 mL sterile distilled water containing 0.1% Tween 20 per liter and filtered through four layers of sterile cheese cloth. Concentration of conidia was determined with a hemacytometer and adjusted to 9×10^7 conidia per mL with sterile distilled water. Inoculum not used immediately was stored at 4°C. Seven days after silks had emerged from 50% of the plants in a plot, the primary ear of each plant was inoculated with 3.4 mL of the conidial suspension injected underneath the husk into the side of the ear using an Indico tree-marking gun fitted with a 14-guage hypodermic needle [25].

For the second inoculation technique, *A. flavus* infected wheat kernels were dispensed into plant whorls. Wheat seeds were infected with *A. flavus* strain NRRL 3357 using a procedure developed by Bock and Cotty [36]. One kilogram of wheat seed and 70 mL distilled water were placed in a 2-liter Nalgene plastic canister and rolled for 20 min on a five-position Wheaton bottle roller apparatus. Canisters containing wheat were autoclaved for 60 min, cooled to room temperature, and autoclaved for an additional 60 min. After cooling to room temperature, 150 mL of a spore suspension containing 1.0×10^6 conidia per mL was added to the wheat. The mixture was rolled for 3 hr to allow for dispersal of the inoculum on the wheat. The canisters were incubated at 29°C for 24 hr. The infected wheat was placed in sterile pillow cases and dried in a greenhouse at ca. 60°C for 48 hr. The wheat was stored at room temperature in closed Nalgene plastic containers until needed for inoculating plants. At 35 and 49 days after planting, each plant in a plot was inoculated with *A. flavus* by dispensing the infected wheat kernels into the whorl of each plant in a plot using a handheld dispenser designed for infesting plants with insects [37]. Two applications of ca. 26 (1 g) wheat kernels per plant were made on each inoculation date.

2.3. Determination of Concentration of Aflatoxin and Fungal Biomass. Primary ears from 10 plants in each plot were hand

harvested at maturity, approximately 63 days after mid-silk, and dried at 38°C for seven days. The ears from each plot were bulked and shelled using an Almaco maize ear sheller (Allan Machine Company, Nevada, IA, USA). Shelled grain from each row was thoroughly mixed by pouring into a sample splitter twice and ground using a Romer mill (Union, MO, USA). The concentration of aflatoxin in a 50 g sample of ground grain was determined by the VICAM Aflatest (Watertown, MA, USA). This procedure can detect aflatoxin at concentrations as low as 1 ng/g.

Aspergillus flavus biomass was determined from the ratio of *A. flavus* DNA (ng) to maize genomic DNA (ng) in each grain sample using quantitative real-time polymerase chain reaction (qRT-PCR) assays [38, 39]. Species specific primers, Af2 amplifying the internal transcribed spacer 1 sequence of *A. flavus* and Zmt3 amplifying maize α-tubulin, were used to quantify *A. flavus* and maize, respectively. The Light Cycler SYBR Green I Master (Roche Applied Science, Germany) was used at 1x concentration with 2 μL of sample template (approximately 10 ng/μL) in 10 μL reaction volumes. The PCR conditions were 95°C for 10 min for initial denaturation, followed by 45 cycles of 95°C for 10 s, 59°C for 5 s, and 72°C for 10 s. The melting curve had one cycle which included 95°C for 10 s and 65°C for 1 min followed by a gradual heating to 97°C with 5 acquisitions per °C. A final cooling step at 40°C for 10 s concluded the PCR protocol. Both *A. flavus* and maize DNA were quantified in each biological sample and the standards, using Af2 and Zmt3, respectively, in separate wells in the same run. Two technical replicates were run for each biological sample and were included in the same run on the same 96-well plate. For DNA quantification, separate standard curves for each set of primers were generated. For Zmt3, several standards with serial dilutions of maize DNA, 40, 30, 20, 4, 0.8, and 0.16 ng/μL, and for Af2 primers, a series of mixed DNA standards containing varying concentrations of *A. flavus* DNA, 10, 1, 0.1, 0.01, 0.001, 0.0001, and 0.00001 ng/μL, in a constant maize DNA concentration of 1 ng/μL were used to construct the standard curve.

2.4. Statistical Analyses. Values for aflatoxin concentrations and ratios of *A. flavus* to maize were transformed as $\ln(y + 1)$, where y is the concentration of aflatoxin or ratio of *A. flavus*/maize in a sample, before statistical analysis. Data were combined for the two years and analyzed using the SAS General Linear Models procedure [40], and the variance was partitioned as appropriate for a split plot arrangement of treatments with inoculation methods as the main plot and hybrids as the subplot. Means were compared using Fisher's protected least significant difference (LSD) at $P = 0.05$ [41]. Following statistical analysis, the transformed means were converted to the original units of measurement (geometric means) to facilitate comparisons between treatments and among hybrids.

3. Results

In the combined analysis of variance for aflatoxin accumulation, years, inoculation methods, and hybrids were highly significant sources of variation ($P = 0.01$). Interactions of years with inoculation methods and with hybrids were also significant ($P = 0.05$). Interactions of inoculation methods × hybrids and years × inoculation methods × hybrids were not significant sources of variation for aflatoxin accumulation. For *A. flavus*/maize ratio, the only significant sources of variation were hybrids ($P = 0.01$) and hybrids × years ($P = 0.05$).

Table 1: Geometric means for aflatoxin accumulation in 10 maize single cross hybrids grown at Mississippi State in 2011 and 2012 following inoculation with *A. flavus* using the side-needle technique or infected wheat kernels.

Single cross hybrid	Aflatoxin (ng/g)	
	Side needle	Wheat
Va35 × PHW79	2190[a]	377[a]
TZAR101 × Seagull 17	959[ab]	295[ab]
Va35 × Seagull 17	791[ab]	286[ab]
Mp04:127 × PHW79	309[bc]	214[abc]
Mp717 × PHW79	561[b]	73[cde]
Mp494 × Seagull 17	115[c]	35[de]
Mp313E × Mo18W	23[d]	21[ef]
Mp313E × Mp715	27[d]	5[g]
Mp313E × Mp717	10[d]	6[fg]
Mean	190	64

Data were transformed ($\ln(y + 1)$, where y = aflatoxin concentration) before analysis. Geometric means were calculated by converting logarithmic means back to the original units of measure. Means in a column followed by the same letter do not differ (Fisher's Protected LSD, $P = 05$).

Overall aflatoxin accumulation was higher in 2011 than in 2012. In each year, side-needle inoculations produced higher levels of aflatoxin than inoculations with infected wheat. Means of hybrids averaged over two years exhibited significant differences with both inoculation methods (Table 1). Mean levels of aflatoxin accumulation for hybrids inoculated with the side-needle technique and with *A. flavus* infected wheat were highly correlated ($r^2 = 0.76$, $P \leq 0.0011$). With both inoculation methods, aflatoxin accumulation was lowest in the single cross hybrids Mp313E × Mp717, Mp313E × Mp715, and Mp313E × Mo18W, whereas it was highest in Va35 × PHW79, TZAR101 × Seagull 17, and Va35 × Seagull 17.

The *A. flavus*/maize ratios were greater in 2011 than in 2012, and the highest mean *A. flavus*/maize ratio was recorded in the side-needle technique. As with aflatoxin accumulation, Mp313E × Mo18W, Mp313E × Mp715, and Mp313E × Mp717 had the lowest ratios, and Va35 × PHW79, TZAR101 × Seagull 17, and Va35 × Seagull 17 had the highest ratios of *A. flavus*/maize with both inoculation techniques (Table 2).

4. Discussion

Although inoculating plants with *A. flavus* infected wheat kernels produced lower levels of aflatoxin accumulation than the side-needle technique, both inoculation methods produced aflatoxin levels that were adequate to differentiate among hybrids with varying levels of resistance to aflatoxin accumulation. The significant correlation between mean aflatoxin accumulation recorded in the two inoculation

Table 2: *Aspergillus flavus*/maize ratio for 10 maize single cross hybrids following inoculation with *A. flavus* using the side needle technique or infected wheat in 2011 and 2012.

Single cross hybrid	*Aspergillus flavus*/maize ratio ($\times 10^{-6}$)	
	Side needle	Infected wheat
Va35 × PHW79	122.4^{ab}	207.8^{a}
TZAR101 × Seagull 17	132.3^{ab}	105.8^{ab}
Va35 × Seagull 17	214.9^{a}	87.4^{ab}
Mp04:127 × PHW79	50.2^{bcde}	66.8^{abc}
Mp717 × PHW79	151.4^{ab}	54.0^{bcd}
GA209 × SC212m	87.9^{abc}	46.1^{bcd}
Mp494 × Seagull 17	79.7^{abcd}	32.2^{bcde}
Mp313E × Mo18W	19.7^{e}	20.1^{cde}
Mp313E × Mp715	27.4^{cde}	12.6^{e}
Mp313E × Mp717	23.5^{de}	18.3^{de}
Mean	70.2	47.9

Data were transformed ($\ln(y + 1)$, where y = ratio of *A. flavus*/maize) before statistical analysis. Geometric means were calculated by converting logarithmic means back to original units of measure. Means in a column followed by the same letter do not differ (Fisher's Protected LSD, $P = 0.05$).

methods and the consistency of aflatoxin accumulation in hybrids observed across the two inoculation methods and years indicate that either should be effective in screening maize germplasm for resistance to aflatoxin accumulation. Although the side-needle technique has been used successfully to evaluate maize germplasm for resistance to aflatoxin accumulation for many years, this technique requires repeated observations of test plots to record dates of silk emergence and to determine when the plants in each plot should be inoculated. Because plots are inoculated 7 days after silks have emerged from 50% of the plants in a plot, multiple passes through the field are required to inoculate all plots in an experiment. On the other hand, inoculation with *A. flavus* infected wheat requires only two passes through the field: one at 35 days after planting and a second at 49 days after planting. The reduction in time required to inoculate field plots with *A. flavus* infected wheat could permit testing of more maize genotypes or added replications without substantially increasing the overall effort during the critical inoculation period. Furthermore, inoculation with *A. flavus* infected wheat does not involve wounding developing ears. Wounding that accompanies use of the side-needle technique could prevent expression of potential resistance traits such as those associated with silks, husk coverage, pericarp thickness, or kernel integrity that impede the entry of the fungus into the ear.

In earlier studies, *A. flavus* biomass and aflatoxin accumulation were positively correlated when maize genotypes varying in levels of resistance were inoculated using the side-needle technique [39]. In this study *A. flavus*/maize ratio (an indicator of fungal biomass) did not differ significantly with the two inoculation methods. However, significant differences were found among hybrids in fungal biomass with both inoculation methods. The *A. flavus*/maize ratio was lowest for the three hybrids that also had the lowest levels of aflatoxin accumulation with both inoculation methods. These results indicate that selection for a lower *A. flavus*/maize ratio should be effective in selecting and developing maize germplasm with resistance to aflatoxin accumulation.

5. Conclusions

Inoculation of plants with *A. flavus* infected wheat was effective in identifying maize genotypes with resistance to aflatoxin accumulation. Although aflatoxin accumulation was lower with this technique than with the side-needle technique, the trends over the two years were similar. This technique does not wound developing ears and thus permits expression of resistance associated with such traits as husk coverage, pericarp thickness, or kernel integrity that would otherwise be masked by the side-needle technique. Savings in time and labor associated with inoculation of plants with *A. flavus* infected wheat are another advantage over the side-needle technique. Additional experiments will be conducted to determine the optimum number and timing of applications of infected wheat for achieving high levels of infection for assessing resistance. The *A. flavus*/maize ratio could be used to complement aflatoxin accumulation as a measure of resistance. Quantifying both aflatoxin and *A. flavus* could be helpful in determining the mechanisms and genetic basis of resistance to aflatoxin accumulation.

Conflict of Interests

None of the authors of this paper has a conflict of interests in terms of the products mentioned. Mention of trade names or commercial products is solely for the purpose of providing specific information and does not imply recommendation or endorsement by USDA.

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