An Inoculation Device to Evaluate Maize for Resistance to Ear Rot and Aflatoxin Production by *Aspergillus flavus*

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ABSTRACT

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Fifteen commercially available maize hybrids were evaluated for variation in susceptibility to *Aspergillus flavus* and aflatoxin production using an inoculation technique that wounds kernels and injects a suspension of *A. flavus* conidia under the husk. The inoculator consists of seven rows of 23 pins mounted in an aluminum bar, with 0.8 cm of the point ends exposed. Located in the center of the pins is a larger needle through which a spore suspension is injected under the husk. The inoculator is mounted at the end of a spray gun which is attached to a backpack sprayer. The inoculator is aligned with the ear axis, the pins are forced through the husk into kernels, and inoculum is injected under the husk. Hybrid rank for ear rot was significantly correlated between inoculations done in 1990 and 1991. Aflatoxin values, however, were not significantly correlated between years. All 15 hybrids were considered moderately to highly susceptible to *A. flavus* ear rot and aflatoxin accumulation. The inoculating device allows for more rapid evaluation of resistance to *A. flavus* ear rot than do some previous techniques. A time-of-inoculation study conducted in 1991 indicated that inoculations at 17, 20, and 23 days after midsilk result in the severest ear rot.

Additional keyword: corn

Kernel rot of maize (*Zea mays* L.), caused by *Aspergillus flavus* Link:Fr., and the subsequent production of aflatoxin is prevalent in the midwestern United States during years with drought conditions. Even though kernel rot due to *A. flavus* is not severe every year, it can create serious problems in the marketing of maize.

The most effective control of *A. flavus* and aflatoxin production in maize is through the development of genetically resistant hybrids. Since the early 1970s, sources of genetic resistance have been identified (8,11-13,15,18,23,25). Because natural infection by *A. flavus* is not consistent enough to evaluate genotypes (4,27), an important aspect of this research is the development of reliable inoculation procedures. To evaluate the large numbers of genotypes needed in a pragmatic breeding program, the inoculation procedure must be rapid and must produce sufficiently high levels of kernel infection to separate susceptible and resistant genotypes.

Previous studies indicate that kernel wounding is necessary to obtain levels of kernel infection and aflatoxin production sufficient to differentiate genotypes. Rambo et al (17) evaluated three inoculation techniques that have been widely used: atomizing a suspension of *A. flavus* conidia onto silks 1-3 wk after silking, injecting a suspension of conidia through the husk into kernels at early milk to dough stage, and inserting a cotton swab dusted with conidia into a hole drilled in the side of the ear. They concluded that kernel wounding was necessary due to the limited parasitic ability of *A. flavus*. Calvert et al (1) compared three techniques for injuring kernels by inoculating maize lines for which pericarp thickness varied. The methods used were: puncturing kernels with a pinboard (sewing pins arranged in rows and mounted on a plexiglass holder), wounding kernels using razor blades mounted in a plastic holder, and wounding kernels with a 25-mm needle mounted in a plastic syringe. Aflatoxin levels were highest with the pinboard or razor blade techniques. Higher amounts of aflatoxin were produced in kernels of genotypes with thin pericarps than in genotypes with thick pericarps. King and Scott (9) evaluated four inoculation techniques, two of which involved kernel wounding. In one method, conidia were injected into individual kernels using a hypodermic syringe fitted with a 0.405-mm-diameter needle. In another method, a pinbar (a single, 100-mm-long row of 35 sewing pins mounted in a plastic bar) was used to wound kernels. The other two inoculation techniques were injecting conidia into the silk channel of the ear and exposing kernels to natural infection by removal of the ear husk leaves. The pinbar technique resulted in the highest (9-48%) kernel infection. Tucker et al (21) evaluated four single-cross hybrids using the pinbar, a knife, exposed kernels, and silk inoculation techniques. Only the pinbar method separated hybrids into groups based on susceptibility to *A. flavus*. Zummo and Scott (29) compared six inoculation techniques. They found that the pinbar and two inoculation techniques that caused very little injury (side needle and needle in the silk channel) resulted in adequate infection for identifying resistance in Mississippi. They preferred the less injurious techniques, because wounding circumvents possible resistance mechanisms of intact kernels. Environments in the midwest often are not conducive to *A. flavus* development; therefore, kernel wounding may be necessary to produce disease levels high enough to separate resistant and susceptible genotypes.

In addition to different inoculation techniques, various kernel sampling procedures have been utilized to obtain sam-

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ples for aflatoxin analyses. In most studies, entire ears are shelled and aflatoxin analyses are made on subsamples of bulked grain containing both inoculated and noninoculated kernels (3,21,25, 27). Although this technique is rapid, ear size differences among genotypes may result in different proportions of inoculated and noninoculated kernels (3). Others have sampled only visibly damaged kernels (5) or nonwounded kernels adjacent to wounded kernels (8,19). These two sampling procedures require more time and labor, which may limit the number of genotypes that can be tested. Analysis of only wounded kernels eliminates variation in aflatoxin content due to ear size, but it may be inappropriate since some mechanisms of resistance may be overcome by severe kernel wounding. Conversely, aflatoxin analysis of only noninoculated kernels adjacent to inoculated kernels does not identify the resistance that may function in wounded kernels.

Previous studies (1,13,14,19,23,24,26) have shown that inoculations 20 days after midsilk (50% of plants in plot with emerged silks) result in the greatest ear and kernel rot and aflatoxin production. Most of these studies (13,14,19,23,25) were conducted in the southern United States.

The objectives of this study were to evaluate a pinboard inoculation technique for screening corn genotypes for resistance to *A. flavus* and aflatoxin in a midwestern environment, to evaluate 15 commercial corn hybrids for resistance to *A. flavus* and aflatoxin, and to compare various dates of inoculation for *A. flavus* ear rot in a midwestern environment.

MATERIALS AND METHODS

In 1990 and 1991, 15 maize hybrids were evaluated for resistance to *A. flavus* and aflatoxin at the Agronomy–Plant Pathology South Farm, Urbana, Illinois. Three hybrids (C2998, Com19, and Com79) were selected based on susceptibility to naturally occurring *A. flavus* and high aflatoxin levels during the drought of 1988 in Illinois (D. G. White, *personal observation*). Four hybrids (Com62, DK614, DK677, and DK689) were selected because of relatively good grain quality. Six hybrids (C6973, C4843, C9979, C6114, C1914, and C8004) were selected by the Independent Professional Seedsmen Association (IPSA) because of their wide use throughout the midwest. Two hybrids (B73 × Mo17 and B73 × LH38) were selected based on their previous use in the midwest.

In 1990, two-row plots (5.34 m long, with row spacing of 0.76 m thinned to 24 plants per row) were planted 2 May in a randomized complete-block design with two replicates. Plants were inoculated approximately 18–22 days after midsilk (DAM). The primary ear of each plant in each plot was inoculated. The inoculation technique consisted of wounding the ear with a pinboard similar to one used by Calvert (1) and injecting 2 ml of spore suspension through the husk in the center of the wounded area. A 50-ml Pistol Grip Syringe (Ideal Instruments, Chicago, IL) fitted with a stainless steel needle similar to that described by Koehler (10) was used to inject inoculum. The pinboard contained seven rows of 17 sewing pins (pins were 2.5 cm long, with 1.0 cm of the point ends exposed, spaced at 0.6 cm) mounted in a 15 × 4 cm plastic bar. The inoculum was prepared in 1990 from the isolate NRRL 6539 (D. T. Wicklow, USDA Northern Regional Research Center, Peoria, IL). Inoculum was produced on potato-dextrose agar in petri dishes incubated at 28 C with 12 hr of light for 12–16 days. Cultures were blended with water and filtered through a double layer of cheesecloth. The resulting spore suspension was adjusted to 2×10^5 conidia per milliliter by dilution with distilled water, and two drops of Tween 20 per 100 ml was added. Inoculum was prepared immediately before inoculation.

In 1991, the 15 commercial hybrids were evaluated at seven times of inoculation. A split-plot design was used with hybrids as the main plot factor and time of inoculation as subplot factors. Main plots were replicated three times, with seven dates of inoculation for subplots. Plots were planted 6 May and consisted of 14 rows (two rows per inoculation date) 5.34 m long, with a row spacing of 0.76 m, with 24 plants per row. Inoculations were started at 14 DAM and continued at 3-day intervals until 32 DAM. Inoculations were done using a pinboard inoculator developed at the University of Illinois (Fig. 1). The pinboard consisted of seven rows of 23 pins (No. 2 rex steel safety pins, American Pin and Fastener Corp., Tempe, AZ). Two centimeters of the point ends were removed and mounted in a slightly concave aluminum bar (16.0 × 4.5 cm) with 0.8 cm of the point ends exposed, and spaced 0.4 cm apart (Fig. 1A). Five milliliters of a spore suspension of 2×10^5 conidia per milliliter was injected under the ear husk through a large needle in the center of the pins. The pinboard was mounted at the end of a MeterJet spray gun (Spraying Systems Co., Wheaton, IL) which metered the inoculum (Fig. 1B). The spray gun was attached to a Solo backpack sprayer (Solo Co., Newport, VA) containing the inoculum. The pinboard was aligned with the ear axis, the pins were pushed through the husk into the kernels, and inoculum was injected under the husk of the primary ear of each plant.

Inoculum was an equal mixture of four isolates of *A. flavus* (NRRL isolates 6536, 6539, and 6540, and an isolate obtained from corn grain in Illinois in 1988). Selection of *A. flavus* isolates was based on high levels of virulence and aflatoxin production from a 1990 isolate virulence study (D. G. White, *unpublished*). Inoculum preparation procedures were the same as in 1990.

Between 40 and 50 days after inoculation, inoculated ears were husked, and a visual rating of 1–10 (1 = 10%, 10 = 100% of the inoculated area rotted) was determined for each ear and averaged for each plot. Ears were harvested from each plot after visual ratings were complete. In 1991, ears only from the 20-DAM inoculation treatment were harvested. In 1990, kernels from within the inoculated area of each ear were removed and bulked for aflatoxin analysis. Due to the extremely high levels of aflatoxin from kernels within the inoculated area in 1990, and the relatively similar ear size of hybrids used in the study, entire ears were shelled and bulked for aflatoxin analysis in 1991. Kernels were ground using a Romer Mill (Model 2A) Grinding/Subsampling Mill, and aflatoxin was analyzed on a random subsample of the ground sample. Samples were extracted (6) and analyzed by high-pressure liquid chromatography (20) at the Veterinary Medicine Center at the University of Illinois, Urbana.

Data were analyzed using SAS software (SAS Institute, Cary, NC). Genotypes and years were assumed to be fixed and random, respectively. Pearson and Spearman rank correlation coefficients were calculated between ear rot and aflatoxin values within years and rank of hybrids for ear rot and for aflatoxin between years, respectively.

RESULTS AND DISCUSSION

Hybrids were significantly different ($P \leq 0.05$) for both ear rot ratings and aflatoxin. The year × genotype interaction was significant for the combined analysis; therefore, the LSD and coefficient of variation (CV) values are based on the error terms from each year. Ear rot ratings and aflatoxin values are listed separately by year (Table 1). Ear rot ratings were higher in 1991 (mean of seven inoculation dates = 5.02) than in 1990 (mean = 3.21). High ear rot severity in 1991 can be explained in part by the higher volume of inoculum (5 ml in 1991 vs. 2 ml in 1990), differences in isolates, and also by the environmental conditions favorable for *A. flavus,* which included higher than average temperatures and lower than average rainfall. The higher aflatoxin concentrations in 1990 were due to different kernel sampling techniques for aflatoxin analyses (analyses of samples from wounded kernels in 1990 vs. analyses of samples from entire shelled ears in 1991).

C2998, Com19, and Com79 had high ear rot ratings and aflatoxin values. These three hybrids also were identified as susceptible to natural infection by *A.*

flavus and aflatoxin production during the drought of 1988 in Illinois (D. G. White, *personal observation*). All 15 commercial hybrids were moderately to highly susceptible to *A. flavus* ear rot and aflatoxin accumulation. This indicates the serious potential for *A. flavus* infection of and aflatoxin accumulation in corn grain grown during drought conditions.

Lower ear rot rating did not always correspond with lower aflatoxin values. Ear rot ratings for C1914 and C8004 were lower than the experimental means in 1990 and 1991; yet aflatoxin values were higher than the experimental mean in both years. Other studies (7,16,21) have reported a high correlation between *A. flavus* kernel infection and aflatoxin accumulation. In this study, Pearson correlation coefficients between ear rot ratings and aflatoxin values were not significant either year. This may be explained by the low number of genotypes included in the study and a narrow range of response among the 15 moderately to highly susceptible genotypes. Spearman's rank correlation coefficient for ear rot rating between years was highly significant ($r_s = 0.78$, $P \geq 0.01$); but the rank correlation was not significant for aflatoxin values. Gardner et al (5) reported a significant ($P = 0.05$) but low ($r_s = 0.45$) Spearman's rank correlation between rank for mean aflatoxin B_1 of 21 diverse F1 crosses between 2 yr where samples for aflatoxin analyses were taken from the inoculated area of the ear in one year and from entire ears in the other year. In our study, the different sampling techniques may have been partially responsible for rank difference of hybrids for aflatoxin between years; however, it is more likely that rank differences were due to the difference in environment between 1990 and 1991. Visual ratings of ears were repeatable assessments of *A. flavus* infection but were a variable measure of aflatoxin production. Due to inherent variability of aflatoxin production, ear rot ratings and aflatoxin assays should be done over several years before classifying genotypes as resistant, moderately resistant, etc. Ear rot and aflatoxin CVs were quite acceptable, especially considering the variability associated with similar studies (4,27). This indicates that our pinboard inoculator is a reliable technique, suitable for screening corn genotypes in the Illinois environment.

The commonly accepted time for ear inoculation has been approximately 20 DAM. Comparing ratings of six hybrids showed ear inoculations on 17, 20, and 23 DAM resulted in higher levels of ear rot than later inoculations (Fig. 2). Rambo et al (17) also found that inoculation at the late milk stage resulted in the highest level of infection with no apparent increase through the early dough stage.

The main advantage of the pinboard technique is its usefulness for screening large numbers of genotypes. The technique allows for sufficient kernel wounding and inoculum introduction throughout the wounded area. To identify resistant genotypes, a large number can be screened for reaction to ear rot, and those with low ratings can be selected for aflatoxin assays, thus saving the expense of aflatoxin analysis. In a separate study done in 1991, 1,189 and 978 inbreds crossed onto Mo17 and B73, respectively, were evaluated for resistance to *A. flavus* ear rot (2). The average ratings of all inbreds crossed with Mo17 and B73 were 5.5 and 5.4, respectively. Thirty-three sources with low levels of *A. flavus* ear rot were selected for further study, and aflatoxin analyses were completed on these selections.

Kernel wounding and inoculum placement in the wounded area may be more critical in the Illinois environment than in the southeastern United States, where conditions often are more conducive to *A. flavus* infection and aflatoxin production (28). Although mechanical wounding of kernels simulates insect damage, wounding circumvents aleurone and pericarp resistance to infection. Since the pinboard method only identifies resistance that functions in the kernel after wounding, pericarp layer and silk channel resistance will be negated. This may cause the incorrect rating of some genotypes as susceptible when they may have sufficient levels of resistance (1,3,22).

Preharvest infection in corn by *A. flavus* is most prevalent under drought conditions. Our screening studies were done under nondrought conditions. It is unclear whether there is an interaction between host resistance to *A. flavus* infection and environmental conditions

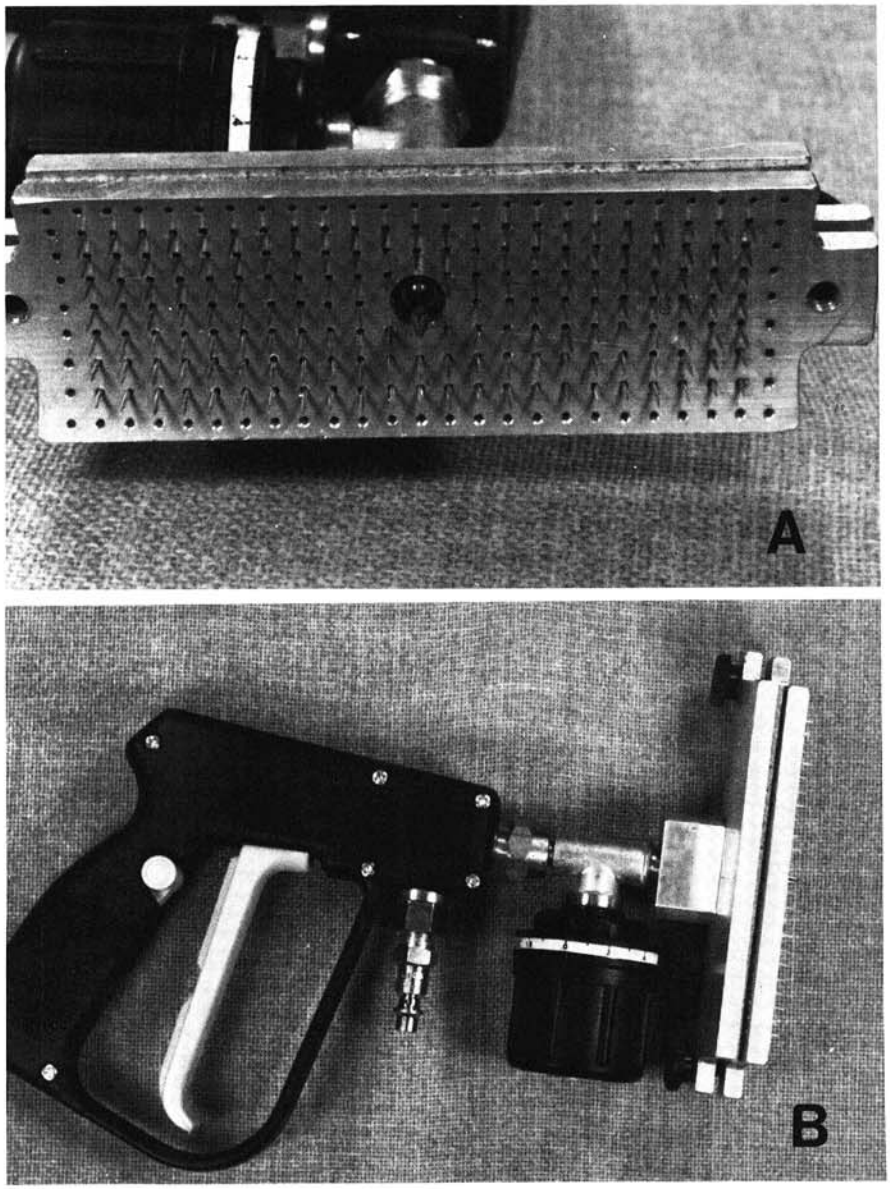

Fig. 1. Inoculation device for screening maize genotypes for *Aspergillus flavus* ear rot and aflatoxin accumulation: **(A)** aluminum pinboard containing seven rows of 23 pins and a large needle through which liquid inoculum was injected under the ear husk, and **(B)** aluminum pinboard mounted on a MeterJet spray gun which metered the inoculum.

which may complicate screening in nondrought conditions.

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Table 1. Ear rot rating and aflatoxin production in grain of corn hybrids following inoculation wih *Aspergillus flavus*

Hybrid	1990 Ear rot rating[a]	1990 Aflatoxin[b] (ng/g) B_1	1990 Aflatoxin (ng/g) B_2	1991 Ear rot rating	1991 Aflatoxin (ng/g) B_1	1991 Aflatoxin (ng/g) B_2
C2998	5.65	5,046	110	5.69	1,739	62
Com19	4.98	3,372	92	5.58	1,708	64
Com62	4.73	6,153	157	5.90	2,058	67
C6973	4.45	5,754	139	5.63	1,290	49
Com79	4.33	7,268	176	6.06	1,611	55
C4843	3.35	9,525	265	5.00	1,569	76
C9979	2.90	4,916	164	4.73	2,582	153
B73 × Mo17	2.80	6,134	164	4.36	1,779	74
DK677	2.55	2,878	81	4.60	1,739	73
B73 × LH38	2.33	2,036	59	4.55	1,514	70
DK614	2.30	5,393	138	5.05	1,754	81
C6114	2.22	2,322	87	4.94	2,297	132
DK689	1.85	2,693	67	4.36	705	26
C1914	1.85	7,301	197	4.66	1,856	68
C8004	1.85	5,231	119	4.24	1,751	79
Mean	3.21	5,068	134	5.02	1,730	76
LSD 0.05	1.60	3,431	75	0.51	814	56
CV (%)	23.21	32	26	16.80	28.13	44.10

[a]Ear rot ratings were on a 1-10 scale, where 1 = 10% and 10 = 100% of the inoculated area rotted. 1990 ratings are based on mean of two replicates of inoculations performed at approximately 20 days after midsilk (DAM), and 1991 ratings are based on mean of seven dates and three replicates of inoculations initiated 14 DAM and continued at 3-day intervals until 32 DAM.

[b]Aflatoxin measured on samples taken from the inoculated portion of the ear in 1990 and on whole ear samples in 1991; 1991 aflatoxin determinations made on inoculations 20 DAM.

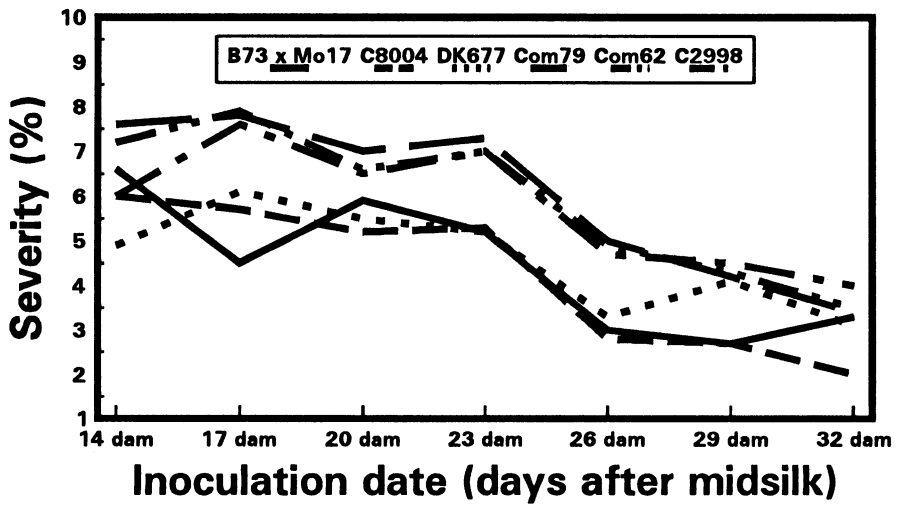

Fig. 2. Severity of *Aspergillus flavus* ear rot on six commercial hybrids inoculated at various days after midsilk in 1991.