Evaluation of Field Inoculation Techniques for Screening Maize Genotypes Against Kernel Infection by *Aspergillus flavus* in Mississippi

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ABSTRACT

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Five inoculation techniques—needle application of inoculum through the silk channel, needle application of inoculum through the husks onto the kernels, toothpick in the ear, toothpick in the silk channel, and string around the silks—were compared with pinbar inoculation for screening maize genotypes against kernel infection by *Aspergillus flavus* in the field. The pinbar inoculation technique generally resulted in higher kernel infection levels than the other inoculation methods but had the disadvantage that some kernels on the ear were injured and hand-shelling of kernels for assay was required. The needle in the silk channel and the needle through the husks produced approximately equal levels of kernel infection without injury to ears or kernels and separated resistant and susceptible maize genotypes. The toothpick in the ear, toothpick in the silk channel, and string around the silk inoculations resulted in significantly less kernel infection than the pinbar and needle inoculations. Increasing conidial concentration in the needle inoculations resulted in greater kernel infection. These studies showed that maize genotypes can be screened in the field for resistance to kernel infection by *A. flavus* using the pinbar, side needle through the husks, and needle in the silk channel inoculation techniques.

Infection in maize (*Zea mays* L.) by *Aspergillus flavus* Link ex Fries and aflatoxin contamination has become a serious problem in the southeastern United States. Feeding maize contaminated with aflatoxin B_1 or B_2 to livestock has resulted in serious losses (1–3,6,17). Toxin production in corn was identified in the early 1960s but was considered to be primarily a stored grain problem until aflatoxin contamination was detected in field samples in 1971 (11,14). In general, aflatoxin contamination is more serious in the southeastern United States than in the corn belt, but it has been reported in the Midwest (9). Available data suggest that few corn genotypes have a measurable level of resistance to *A. flavus* (9,10,12,13,16,18).

A major difficulty in establishing priorities for research on *A. flavus* has been that the fungus actually causes little, if any, measurable losses of corn yields directly. The magnitude of the *Aspergillus*/aflatoxin problem generally becomes apparent only when the effects of feeding grain contaminated with high levels of aflatoxin are observed. Aflatoxin in livestock and poultry feed can cause serious problems, including dramatic weight loss and death of affected animals (2,5,6,17). The United States Food and Drug Administration prohibits interstate shipment of corn containing more than 20 ppb of aflatoxin.

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There is need for a reliable inoculation technique that will result in a sufficiently high level of kernel infection by *A. flavus* in susceptible genotypes to allow identification of genotypes with resistance. Much of the field-screening research has been patterned after the three techniques of inoculation used by Rambo et al (15): 1) atomizing a conidial suspension onto the silks 1–2 wk after silking, 2) injecting a conidial suspension through the husk into kernels at the early milk to early dough stage with a hypodermic syringe, and 3) inserting a cotton swab dusted with conidia into a hole drilled into the ear. They concluded that *A. flavus* has limited parasitic ability and that wounding is probably needed for kernel infection. Widstrom et al (19) tested three inoculation techniques but failed to find differences among hybrids for aflatoxin contamination, insect damage to ears, or percentage of ears with visible *A. flavus*. King and Scott (8) tested four inoculation techniques for infection of maize hybrids by *A. flavus*; two methods, silk channel and exposed kernel, did not injure kernels, whereas kernel injection and pinbar inoculation resulted in some kernel injury. They considered the pinbar technique best because it resulted in higher levels (9–48%) of kernel infection, was relatively easy to use, and provided a large number of kernels for assay. Tucker et al (18) tested the pinbar, knife, exposed kernel, and silk inoculation methods on four maize hybrids for kernel infection percentage and aflatoxin concentration. Only the pinbar technique permitted complete separation of hybrids into groups based on the relative susceptibility to kernel infection by *A. flavus*. Hybrids Mp428 × SC212M and GT106 × SC343 were grouped as susceptible to kernel infection and hybrids Mo18 × Mp412 and Mp490 × Tx601 were considered resistant (18).

Zuber et al (20,21) and Gardner et al (7) indicated that aflatoxin production in maize is under genetic control. Darrah et al (4), in 1987, reported that genetic control of aflatoxin contamination in maize obtained by kernel wounding using a pinboard (7) was not the same as that obtained in the absence of kernel wounding. Scott and Zummo (16), using inoculation techniques evaluated earlier (22), identified sources of resistance in maize to kernel infection by *A. flavus* in the field. Genotypes classed as susceptible or resistant remained significantly different from each other when inoculated in the field by three different inoculation techniques. The identity of susceptible and resistant maize genotypes now permits the use of some of these genotypes as checks in inoculation experiments. The incidence of kernel infection in test genotypes obtained with different inoculation techniques can now be compared with the incidence in genotypes of known reaction.

The object of this study was to develop a reliable nondamaging method for inoculating maize ears in the field that could be used to evaluate genotypes for relative resistance to kernel infection by *A. flavus*.

MATERIALS AND METHODS

Maize plants were grown in single-row plots 5.0 m long and 1.0 m apart with 20 plants per row. Plots were arranged in a randomized block design, except for the 1986 test, which was a split-plot design with inbreds as whole plots and inoculation technique as the split. All studies were done at the Plant Science Farm, Mississippi State, Mississippi, and treatments were replicated four to six times.

Only the top ear of each plant was inoculated at designated times after the midsilk growth stage (50% of ears with emerged silks for plants in each plot). Ears to be assayed were harvested 60 days after midsilk. Ears from plants in replicated noninoculated plots, harvested 60 days after midsilk, were used as checks. Ears were dried immediately after harvest at 42 C for 7 days in a forced-air dryer.

Inoculum. Inoculum was prepared from a lyophilized culture of *A. flavus* (NRRL 3357) obtained from D. T. Wicklow, Northern Regional Research Center, Peoria, Illinois, each year. Inoculum for 1984 and 1985 was grown on Czapek solution agar (CSA) in petri dishes at 28 C. After 12–14 days, conidia were washed from the surface of the agar with sterile distilled water containing two drops of Tween 20 per 100 ml. Inoculum for 1986 and 1987 was grown on 40-mesh corncob grits (Grit-O'-Cobs, Maumee, Ohio) in 500-ml Erlenmeyer flasks, each containing 50 g of grits and 100 ml of H_2O. After 12–14 days, conidia were washed from the surface of the corncob grits with sterile distilled water containing two drops of Tween 20 per 100 ml. All inoculum for each year's inoculations was prepared at one time and kept at 4 C as an aqueous suspension of 10^8 conidia per milliliter. Inoculum was diluted to the desired concentration on each day of inoculation and kept on ice in the field until used. The viability of the inoculum was tested periodically on CSA. Germination of conidia remained above 90% throughout these studies.

Inoculation techniques. The six techniques compared in this study were: 1) pinbar, 2) needle inoculation into silk channel, 3) side needle inoculation, 4) toothpick in ear, 5) toothpick in silk channel, and 6) string on corn silks. The pinbar inoculation technique used was that described by King and Scott (8) in which a single 100-mm-long row of 35 sewing pins mounted in a plastic bar with 6 mm of the point ends exposed was dipped into a conidial suspension, lined up with the ear axis, and pressed through the husk and into the kernels beneath. Only the rows of kernels adjacent to the pinbar-inoculated row were assayed. The incidence of kernel infection for each plot inoculated with the pinbar was based on a sample of approximately 32 kernels per ear adjacent to the damaged row of kernels on 20 ears compared with a sample from all kernels on the ear with all other inoculation techniques.

The silk channel technique utilized a 14-gauge hypodermic needle, 50 mm long, with the tip opening plugged and three 1-mm holes drilled 6, 8, and 10 mm from the tip. Six days after midsilk, the needle was inserted into the silk channel and a given amount of inoculum was injected inside the husks onto the kernels. A Cornwall continuous syringe-pipette calibrated to deliver 1.0, 2.0, or 3.0 ml of inoculum was used in all needle inoculation tests in 1984 and 1985. The syringe-pipette proved too fragile for routine field use, so in our 1986–1987 experiments we obtained comparable results with an Idico tree marking gun (Idico Products Co., New York, NY) that delivered the suspension in 1.7-ml amounts. When a marker dye was used in the silk channel and side needle inoculations with 3.4 ml of inoculum per ear, over 50% of kernels on each ear came in direct contact with inoculum.

The tree marking gun fitted with a 14-gauge needle 35 mm long, with a sharp plugged point and with three 1-mm holes as described above, was used for the side needle inoculations. Six days after midsilk, the needle was inserted at an angle through the husks and the inoculum was injected over the kernels without damaging them.

For the toothpick techniques, 500 toothpicks, 35 mm long and 2 mm in diameter, were boiled in distilled H_2O for 30 min, rinsed in distilled water, then placed in 1-L Erlenmeyer flasks with cotton plugs and autoclaved for 20 min. The toothpicks were inoculated with 10 ml of an aqueous suspension of 2×10^6 conidia per milliliter of *A. flavus* and incubated for 14 days at 28 C. Using a hemacytometer, it was estimated that 1.7×10^6 spores were recovered from each toothpick. Six days after midsilk, infested toothpicks were inserted through the husks and into the middle portion of the ear or into the silk channel.

For the string technique, pieces of white cotton twine 2 mm in diameter and 100 mm long were boiled in distilled H_2O, arranged concentrically in glass petri plates (50 strings per plate), and autoclaved 20 min. Then, 5 ml of CSA was added to each plate, inoculated with 2 ml of an aqueous suspension of 2×10^6 conidia per milliliter of *A. flavus*, and incubated at 28 C for 14 days. A hemacytometer was used to estimate that an average of 5.4×10^7 spores were recovered from each string. Six days after midsilk, maize ears were inoculated by tying a single string around the silks.

Inoculations. Experiments were carried out in 1984, 1985, 1986, and 1987 to evaluate the kernel inoculation methods for separating resistant and susceptible maize genotypes.

Table 1. Percentage of kernels from 10 maize hybrids infected with *Aspergillus flavus* after inoculation by five techniques in the field at Starkville, Mississippi, in 1984

	Kernels infected (%)					
Hybrid	Pinbar technique	Needle in silk channel technique	Toothpick in ear technique	Toothpick in silk channel technique	String on silks technique	Mean
Mp313E × Mp68:616	2.1	2.9	0.9	0.4	0.9	1.4
Mp77:357 × Mp68:616	4.3	7.2	0.7	0.5	0.7	2.7
Mo18W × SC229	5.2	6.7	0.4	1.1	0.4	2.7
SC54 × SC76	4.8	6.7	0.8	2.0	1.5	3.2
SC212M × Mp307	9.1	11.2	0.8	1.4	1.2	4.8
SC376 × Mp428	10.4	10.9	0.8	2.0	0.7	5.0
Mp317 × Tx601	11.9	11.6	1.3	1.1	1.1	5.4
T232 × GA209	13.4	10.5	1.8	1.6	1.0	5.7
Mp317 × Mp307	15.8	12.0	0.8	0.5	2.2	6.2
Pioneer Brand 3369A	15.3	14.1	11.9	4.2	6.5	10.4
Mean	9.2	9.4	2.0	1.5	1.6	

LSD (0.05) treatment × hybrid means = 4.7
LSD (0.05) among hybrid means = 2.1
LSD (0.05) among inoculation techniques = 1.3

Table 2. Average percentage of kernels from two maize hybrids infected with *Aspergillus flavus* after inoculation by the pinbar technique or by the silk channel (syringe-pipette) technique, using nine conidial concentrations, in the field at Starkville, Mississippi, in 1984

Inoculation technique	Inoculum concentration (conidia/ml)	Inoculum volume (ml)	Time of inoculation (days after midsilk)	Kernels infected (%)
Silk channel[y]				
C1	10^4	2	6	6.73[z]
C2	10^5	2	6	6.22
C3	10^6	2	6	12.14
V1	10^5	1	6	4.35
V2	10^5	2	6	6.46
V3	10^5	3	6	10.30
D1	10^5	2	7	7.68
D2	10^5	2	14	3.66
D3	10^5	2	21	5.79
Pinbar	...	...	20	9.19
Check (not inoculated)	...	...	...	2.40
LSD (0.05)				3.52

[y] C = conidial concentration, V = volume of inoculum, and D = days after midsilk.
[z] Each value is mean percentage of kernels infected with *A. flavus* in six replications of 390 kernels after surface-sterilization and 7 days of incubation at 28 C on Czapek solution agar amended with 7.5% NaCl.

In 1984, the needle in silk channel, pinbar, toothpick in ear, toothpick in silk channel, and string on silks inoculation methods were compared in the field on 10 maize hybrids. Each treatment was replicated four times on each hybrid. Pioneer Brand 3369A, a widely grown commercial hybrid, was included to represent what appeared to be a hybrid susceptible to kernel infection by the fungus.

In 1985, in order to determine the most effective conidial concentration per milliliter of inoculum, inoculum volume, and date of inoculation, the silk channel inoculation technique using the syringe-pipette was compared with the pinbar method on maize hybrids AB24E × Va35 and Pioneer Brand 3369A. Three conidial concentrations (2 ml each of 10^4, 10^5, and 10^6 conidia per milliliter), three inoculum volumes (1, 2, and 3 ml of inoculum containing 10^5 conidia per milliliter), and three dates of inoculation (2 ml of inoculum with 10^5 conidia per milliliter applied at 50% midsilk + 7, 14, and 21 days) were used.

In 1986, six replications of 10 inbred maize genotypes were inoculated with the pinbar, silk channel (3.4 ml of inoculum containing 9×10^6 conidia per milliliter), and side needle (3.4 ml of inoculum containing 9×10^6 conidia per milliliter) techniques.

In 1987, two maize hybrids from susceptible × susceptible inbred lines as described by Scott and Zummo (16) and two hybrids from resistant × resistant inbred lines were compared using the pinbar and side needle (3.4 ml of inoculum containing 9×10^6 conidia per milliliter) techniques. Each treatment was replicated four times. The side needle technique was used because it was easier and resulted in less stalk and ear breakage than the needle in the silk channel method.

A resistant × resistant (Mp313E × SC54) and a susceptible × susceptible (GT106 × SC212M) single cross, as described by Scott and Zummo (16), plus a commercial hybrid (Pioneer Brand 3369A) were planted on 16 April and 15 May 1987, with six replications at each planting date. Treatments were pinbar inoculation of plants 20 days after midsilk and side needle inoculation using three conidial concentrations (3.4 ml of inoculum containing 3×10^6, 6×10^6, and 9×10^6 conidia per milliliter per ear) 6 days after midsilk.

Assay. Kernels that apparently were not damaged during inoculation were assayed for infection by *A. flavus*. Kernels were surface-sterilized by being dipped in 70% ethanol, soaked for 3 min in 1.5% NaOCl, and rinsed in sterile distilled water. The kernels were placed in 100-mm dishes (13 per dish) on CSA amended with 7.5% NaCl to restrict growth of other fungi and bacteria. After 7 days at 28 C, kernels were examined for incidence of *A. flavus* growth. Data were subjected to a standard analysis of variance using the percentage of 390 kernels infected with *A. flavus* as a plot mean. The means in Tables 1, 2, and 3 were separated using LSD, and the means in Tables 4 and 5 were separated using Duncan's multiple range test.

RESULTS AND DISCUSSION

The needle in the silk channel (syringe-pipette) and the pinbar inoculation techniques produced sufficiently high levels of kernel infection in 1984 so that maize hybrids could be separated for response to infection by *A. flavus* (Table 1). The toothpick in the ear, toothpick in the silk channel, and string on the silks inoculation methods resulted in significantly lower incidence of kernel infection than the pinbar or needle method (Table 1). In addition, these methods did not cause differential responses among the maize hybrids tested and consequently were not used in later tests.

Increasing conidial concentration in the needle inoculation technique from 10^4 to 10^6 conidia per milliliter resulted in higher incidence of kernel infection (Table 2). However, increasing conidial concentration from 3×10^6 to 9×10^6 conidia per milliliter of inoculum did not significantly increase kernel infection (Table 3).

The needle in the silk channel, side needle, and pinbar inoculation techniques produced sufficient differential response among maize genotypes tested to enable differences in resistance to kernel infection by *A. flavus* to be determined (Tables 4 and 5).

The side needle technique was the easiest to apply in the field and produced adequate levels of kernel infection using 3.4 ml of inoculum containing 9×10^6 conidia per milliliter. The needle in the silk channel technique was more difficult to apply, especially on corn genotypes with erect ears, because ears had to be parallel with the soil surface during inoculation, resulting in broken ear shanks in some maize genotypes. The needle in the silk channel was, nevertheless, faster and easier to apply than the pinbar technique.

The pinbar technique consistently resulted in higher kernel infection levels than the two needle techniques but had the disadvantage of injuring some kernels on the ear and requiring hand-shelling of kernels for assay. The two needle methods did not result in injured kernels

Table 3. Average percentage of kernels from three maize hybrids infected with *Aspergillus flavus* after inoculation by the pinbar technique or by the side needle technique, using three conidial concentrations, in the field at Starkville, Mississippi, in 1987

	Kernels infected (%)				
		Side needle technique (conidia/ml)			
Hybrid	Pinbar technique	3×10^6	6×10^6	9×10^6	Mean
Mp313E × SC54	11.3	5.6	5.6	5.7	7.0
GT106 × SC212M	31.7	17.7	27.6	23.6	25.2
Pioneer 3369A	27.9	14.4	17.1	16.7	19.0
Mean	23.6	12.6	16.8	15.3	

LSD (0.05) treatment × hybrid means = 8.0
LSD (0.05) among hybrid means = 4.0
LSD (0.05) among inoculation techniques = 4.6

Table 4. Percentage of kernels from 10 inbred lines of maize infected with *Aspergillus flavus* after ear inoculation by the pinbar, silk channel, and side needle techniques in the field at Starkville, Mississippi, in 1986

	Kernels infected (%)		
Inbred	Pinbar technique	Silk channel technique	Side needle technique
SC212M	77.48 a[z]	58.32 a	66.18 a
T216	47.63 b	30.10 b	29.02 b
Mp68:616	40.00 bc	18.00 bc	21.58 bc
GA209	39.63 bc	24.65 bc	29.18 b
T202	36.25 bcd	21.15 bc	17.17 bc
Mo18W	25.33 de	9.73 bc	11.93 bc
SC54	17.68 de	8.92 c	9.42 c
Mp420	16.05 e	7.82 c	7.58 c
Tx601	13.17 e	18.45 bc	8.60 c
Mp313E	11.88 e	5.12 c	4.08 c
Mean	32.51	20.23	20.47

[z] Each value is mean percentage of kernels infected with *A. flavus* in six replications of 390 kernels after surface-sterilization and 7 days of incubation at 28 C on Czapek solution agar amended with 7.5% NaCl. Values in a column not followed by the same letter differ significantly ($P = 0.05$) from each other according to Duncan's multiple range test.

Table 5. Percentage of kernels from four maize hybrids infected with *Aspergillus flavus* after inoculation by the pinbar and side needle techniques in the field at Starkville, Mississippi, in 1987

Hybrid	Kernels infected (%)	
	Pinbar technique	Side needle technique
Mo18 × Mp313E	7.7 a[z]	3.4 a
SC54 × Tx601	15.4 ab	4.5 a
Mp68:616 × SC212M	35.8 b	20.8 b
Gt106 × T202	29.2 b	17.5 b

[z] Each value is mean percentage of kernels infected with *A. flavus* in four replications of 390 kernels after surface-sterilization and 7 days of incubation at 28 C on Czapek solution agar amended with 7.5% NaCl. Values in a column not followed by the same letter differ significantly ($P = 0.05$) from each other according to Duncan's multiple range test.

and the ears could be shelled mechanically. The toothpick in the ear, toothpick in the silk channel, and the string on the silks methods did not result in high enough levels of infection to determine relative resistance of maize genotypes.

The results from kernel infection assays in these studies indicate that maize genotypes can be screened in field tests for resistance to kernel infection by *A. flavus* using the pinbar, side needle, or needle in the silk channel inoculation technique. Maize hybrids containing Mp313E had the lowest levels of kernel infection of all hybrids tested (Tables 1, 3, and 5) and maize hybrids containing SC212M had the highest (Tables 3 and 5). This supports the earlier work of Scott and Zummo (16) in which they identified resistant and susceptible maize genotypes using these three inoculation techniques.

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