Field Inoculation Techniques to Evaluate Maize for Reaction to Kernel Infection by *Aspergillus flavus*

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ABSTRACT

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Four inoculation techniques for evaluating reaction of maize to infection of kernels by *Aspergillus flavus* were tested in the field on 10 commercial maize hybrids representing a range of kernel hardness. Inoculations were made 20 days after the midsilk stage, and at maturity kernels not wounded during inoculation were surface sterilized and plated to determine the percentage infection. Inoculation by injecting an *A. flavus* conidial suspension into the silk channel or by atomizing a conidial suspension onto exposed kernels resulted in infection levels too low (≤7%) to adequately differentiate among genotypes. Kernel injection and pinbar inoculation techniques, in which kernels near wound-inoculated kernels were assayed, demonstrated significant differences among some hybrids. The pinbar technique was considered 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. Kernel hardness was not correlated with reaction to kernel infection.

Additional key words: aflatoxin, corn, *Zea mays*.

The association of *Aspergillus flavus* Link ex Fr. with preharvest maize (*Zea mays* L.) was first reported over 60 yr ago by Taubenhaus (17). However, it was not known until after 100,000 turkeys died in 1960 that this fungus was capable of producing a carcinogenic metabolite, later called an aflatoxin (8). Considerable research on the subject ensued, and in maize, it was believed that *A. flavus* infection and aflatoxin production were of consequence only after harvest (2). In the early to mid-1970s, however, it became apparent that aflatoxin contamination was a major problem in maize before harvest in the southeastern United States (1,11,12,21). The potential magnitude of this problem became clear in 1977. A survey of field-collected maize in Georgia indicated that 90% of the samples were contaminated with aflatoxin in excess of 20 ppb (14). Furthermore, it was estimated that over 90% of the 1977 preharvest maize crop in the southeastern USA was contaminated with aflatoxin, with many samples having aflatoxin in excess of 1,000 ppb (25). It has been reported that maize produced in 1980 was again severely affected in some southeastern states, especially North Carolina (G. A. Payne, *personal communication*).

Preharvest infection of maize kernels by *A. flavus* was once thought to depend on kernel wounding such as that caused by insect feeding (4,9,16,17,22). However, it is now apparent that infection by *A. flavus* and subsequent aflatoxin contamination of preharvest maize is not necessarily a function of insect damage (5), although insect damage often enhances these processes.

Host plant resistance has been an effective method of disease control in maize (19), and there is some evidence indicating that maize genotypes differ in production of aflatoxin (9,10, 13,15,23,24). These studies have relied on mechanical injury to kernels at the time of inoculation. The compelling reason for using mechanical injury at the time of inoculation is that *A. flavus* is a weak pathogen with apparently limited ability to infect kernels. However, inoculation by mechanical injury in screening for resistance has the distinct disadvantage of eliminating a possible mechanism of resistance to fungal entry into the kernel, namely the pericarp.

The purpose of the present investigation was to test several inoculation techniques to find one that may be useful in the field in a screening and breeding program for resistance to kernel infection by *A. flavus*. Such a technique must include: an assay for *A. flavus* infection in those kernels not damaged during the inoculation process; and production of sufficiently high levels of kernel infection to differentiate reactions among genotypes. Because nothing is now known on genotypic response in maize to infection of kernels by *A. flavus*, we chose to conduct our study using hybrids that represent a range of kernel hardness, a character we thought might be related to response to kernel infection.

MATERIALS AND METHODS

Four inoculation techniques were tested on 10 commercial maize hybrids (Table 1) representing a range in kernel hardness (based on apparent amount of corneous starch in the endosperm). Plants were grown in plots on the Plant Science Farm, Mississippi State, MS, in 1978, 1979, and 1980 in a randomized block design with three replications. Plots were single, 5-m rows 1 m apart with 15–20 plants per plot.

The primary ear (top) of each plant was inoculated and harvested at 20 and 60 days, respectively, after the midsilk stage (50% of ears with emerged silks) for plants in the plot. Ears from uninoculated control plots also were harvested 60 days after the midsilk stage. Ears were dried at ~42 C for 7 days in a forced-air drier.

Inoculum. Inoculum was prepared as a suspension of conidia of *A. flavus* (NRRL 3357). Cultures were 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 (polyoxyethylene sorbitan monolaurate) per 100 ml. All inoculum for a given 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

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diluted to 2.0×10^7 conidia per milliliter on each day of inoculation and kept on ice until used. An aqueous suspension of *A. flavus* conidia was maintained at 4 C and used as a stock culture for conidial increase each year.

Inoculation techniques. *Silk channel.* One milliliter of conidial suspension was injected into the silk channel by using a Cornwall syringe-pipet equipped with a 50-mm-long, 1.83-mm-diameter (13-gauge), side-delivery needle. After being dried, the ears were shelled, the grain within each plot was bulked, and a random sample of 260 kernels was assayed for *A. flavus* infection.

Exposed kernel. The husk on one side of the ear was peeled back to expose ~25% of the kernels, which were then sprayed with ~1 ml of a conidial suspension. Husks were repositioned, secured with rubber bands, and ears were covered with Kraft paper pollinating bags to prevent bird damage. Bags were left on ears until harvest. After being dried, the ears were shelled, the grain was bulked, and a random sample of 260 kernels was assayed for *A. flavus* infection.

Kernel injection. A 2–3 μl amount of a conidial suspension was injected through the husk and into a kernel by using a threaded-plunger syringe (Hamilton Co., Reno, NV 39510) fitted with a 12.5-mm-long, 0.405-mm-diameter (26-gauge) needle with a partially bent tip that reduced orifice plugging by husk tissue. Each ear was inoculated at four points corresponding to the four corners of a 4×8 cm rectangle with the long sides parallel to the ear axis. After drying, each inoculated kernel and the four closest kernels in both directions from the inoculated kernel in the kernel row were removed and kept separately according to position (Fig. 1). Within each plot, all kernels for a given position were bulked and a random sample of 40–50 kernels per position was assayed for infection by *A. flavus*.

Pinbar. 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 kernels beneath. After drying, the row of inoculated kernels and the two rows of kernels on both sides of the inoculated row were removed and kept separately according to row position (Fig. 2). Kernels from each position in a plot were bulked and a random sample of 100 kernels per position was assayed for infection by *A. flavus*. The estimated amount of visible *A. flavus* growth on the row of inoculated kernels was estimated for each ear on a 0 to 5 scale with 0 indicating no visible growth and 5 indicating complete coverage of kernel caps by growth of *A. flavus*.

Assay. Kernels not physically damaged during inoculation were assayed for infection by *A. flavus*. Kernels were surface sterilized by dipping once in 70% ethanol, soaking for 3 min in 1.6% NaOCl (Clorox), and rinsing in sterile, distilled water. They were placed in standard-sized petri dishes (up to 13 kernels per dish) on CSA amended with 7.5% NaCl to restrict growth of other fungal contaminants and bacteria. After 7 days of incubation at 28 C, plates were examined for *A. flavus* growth and percentage of *A. flavus*-infected kernels was calculated. Data were subjected to a standard analysis of variance.

RESULTS

Percentage *A. flavus* kernel infection in uninoculated control plots did not exceed 1% for any of the 10 hybrids in any of the 3 yr of testing. No significant differences ($P = 0.05$) in kernel infection from natural inoculation were detected among hybrids.

Infection levels following inoculation in the silk channel did not exceed 2.3% for any of the 10 hybrids in any of the 3 yr, and no significant differences ($P = 0.05$) in reaction to kernel infection were

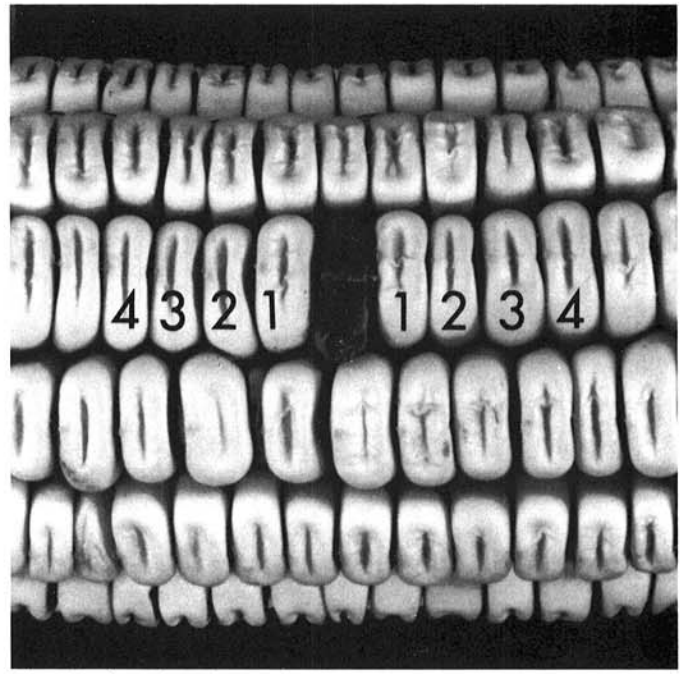

Fig. 1. Portion of a maize ear at harvest indicating the positions of kernels 1, 2, 3, and 4 that were assayed for *Aspergillus flavus* infection relative to the dark infected kernel that had been inoculated by the kernel injection technique.

TABLE 1. Percentage of maize kernels infected with *Aspergillus flavus* following ear inoculation by the kernel injection technique in 1978 and 1980

Hybrid	Brand name	Kernel hardness	Kernel position[w] 1978 1	1978 2	1978 3	1978 4	1980 1	1980 2	1980 3	1980 4
55	Dixie	1[x]	17 [y]b[z]	1 c	1 d	0 d	39 abc	17 abc	16 a	15 abc
18	Dixie	3	25 ab	9 ab	7 abc	4 bc	41 abc	15 bc	11 a	11 abc
XL81	DeKalb	1	23 ab	14 ab	4 bcd	5 bc	48 abc	9 c	14 a	9 bc
XL394	DeKalb	2	27 ab	8 abc	1 d	2 cd	49 abc	24 ab	12 a	15 abc
1295	DeKalb	3	37 a	15 a	11 a	9 a	56 a	19 abc	20 a	20 ab
76-435	DeKalb	4	24 ab	9 ab	9 ab	4 bc	46 abc	20 abc	18 a	15 abc
3147	Pioneer	1	29 ab	13 ab	7 abc	3 bcd	37 bc	10 c	9 a	5 c
3184	Pioneer	2	26 ab	11 ab	5 bcd	6 ab	53 ab	19 abc	15 a	13 abc
3030	Pioneer	3	28 ab	7 bc	3 bcd	1 cd	32 c	26 ab	16 a	16 abc
UC5-2533	Pioneer	4	23 ab	9 ab	2 cd	1 cd	57 a	30 a	21 a	24 a
Mean			26	10	5	4	46	19	15	14

[w] Kernel position refers to location of kernel in the kernel row with respect to the single inoculated kernel. Position 1 = kernel adjacent to inoculated kernel; position 4 = kernel that is four kernels away from inoculated kernel.

[x] Kernel hardness scale: 1 = soft kernel; 4 = very hard kernel.

[y] Each datum is the mean percentage of kernels with *A. flavus* in three replications (40–50 kernels per replication) after surface sterilization and 7 days of incubation at 28 C on Czapek solution agar amended with 7.5% NaCl.

[z] Values within a column not followed by the same letter differ significantly ($P = 0.05$) according to Duncan's multiple range test.

found among hybrids. Mean infection levels from silk channel inoculation were 1.2, 1.2, and 1.3% for 1978, 1979, and 1980, respectively.

Inoculation of exposed kernels was tested in 2 yr. In 1979, mean infection of kernels by *A. flavus* for all 10 hybrids combined was 1.7% with a range of 0.7–2.7% among hybrids. In 1980, the mean kernel infection for all hybrids was 4.5% with a range of 2.1–7.1% among kernels. Statistically significant differences among hybrids were obtained each year, but agreement between years was not good.

The kernel injection technique was tested in 1978 and 1980 (Table 1). Mean *A. flavus* kernel infection levels at kernel positions 1, 2, 3, and 4 (Fig. 1) were 26, 10, 5, and 4%, respectively, in 1978. Dixie 55 had the least infection at all four positions and these values were significantly lower than values for the same positions on DeKalb 1295, the hybrid with the highest levels of infection. In 1980, infection levels at all positions were considerably higher with values of 46, 19, 15, and 14% at positions 1, 2, 3, and 4, respectively.

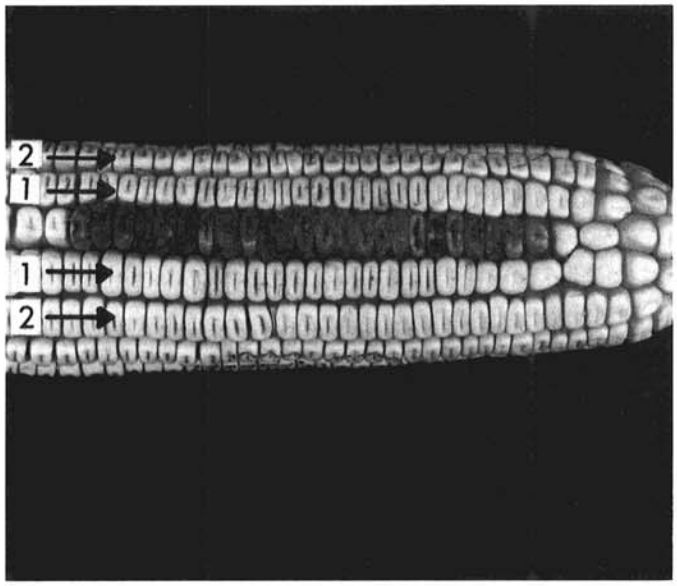

Fig. 2. Maize ear at harvest indicating positions of kernel rows 1 and 2 that were assayed for *Aspergillus flavus* infection relative to the dark row of infected kernels that had been inoculated by the pinbar technique.

TABLE 2. Percentage of maize kernels infected with *Aspergillus flavus* following ear inoculation by the pinbar technique in 1980

Hybrid	Brand name	Kernel hardness	Kernel row[w] 1	Kernel row[w] 2
55	Dixie	1[x]	15 ef[z]	6 cd
18	Dixie	3	20 def	8 bcd
XL81	DeKalb	1	14 ef	7 bcd
XL394	DeKalb	2	37 abc	18 b
1295	DeKalb	3	48 a	39 a
76-435	DeKalb	4	39 abc	14 bcd
3147	Pioneer	1	30 bcd	15 bc
3184	Pioneer	2	26 cde	14 bd
3030	Pioneer	3	9 f	3 d
UC5-2533	Pioneer	4	42 ab	14 bcd
Mean			28	14

[w] Kernel row refers to position of row of kernels assayed with respect to inoculated row. Row 1 = row of kernels adjacent to inoculated row; row 2 = row of kernels that is two rows away from inoculated row.

[x] Kernel hardness scale; 1 = soft kernel; 4 = very hard kernel.

[y] Each datum is the mean percentage of kernels with *A. flavus* in three replications (100 kernels/replication) after surface sterilization and 7 days incubation at 28 C on Czapek solution agar amended with 7.5% NaCl.

[z] Values within a column not followed by the same letter differ significantly ($P = 0.05$) according to Duncan's multiple range test.

Pioneer Brand UC5-2533 had the highest levels of infection at all positions, but the lowest infection value for each position varied among hybrids. All of the kernels that were punctured during inoculation had either disintegrated by harvest or were found to be infected by *A. flavus*.

The pinbar inoculation technique was tested in 1980 (Table 2). Mean infection at kernel row position 1 (Fig. 2) was 28% with a range of 9–48% among hybrids and at kernel row position 2 it was 14% with a range of 3–39% among hybrids. Pioneer Brand 3030 had the least infection with values of 9 and 3% at the two positions and DeKalb 1295 had the most infection with 48 and 39%. Ratings indicating the relative amount of visible *A. flavus* growth on the inoculated row of kernels averaged 3.9 and ranged from 2.8 to 4.7. The amount of this growth was not noticeably correlated with the percentage of kernels infected with *A. flavus* in the two kernel row positions assayed. Assays of kernels punctured during inoculation by the *A. flavus* contaminated pins were all positive for infection by *A. flavus*.

Kernel hardness did not noticeably influence reaction of hybrids to kernel infection, regardless of the inoculation technique used.

DISCUSSION

The silk channel and exposed kernel inoculation techniques did not yield sufficiently high levels of *A. flavus* infection to differentiate among maize genotypes. Our inoculations were made 20 days after the midsilk stage. Infection levels might have been higher had inoculations been made at an earlier or later stage of kernel development. Other studies indicate that highest levels of aflatoxin accumulation are associated with inoculation of the late-milk to early-dough (5,16) or hard-dough (18) stages. We inoculated only about one-fourth of the kernels on the ear with the exposed kernel technique, and then obtained the sample of kernels for assay after shelling all kernels. Percentage kernel infection might have been higher if more of the ear had been inoculated or if only kernels from the inoculated portion of the ear had been assayed. Also, because high humidity is thought to favor infection (5), covering ears with plastic bags for a few days immediately following inoculation might have increased infection levels.

The kernel injection and pinbar inoculation techniques were based on the premise that *A. flavus* would move farther from the site of wound inoculation in susceptible kernels than in resistant kernels. The kernel injection technique gave relatively high levels of infection in position one in both years, with generally decreasing levels in positions farther from the inoculated kernel (Table 1). However, this technique did not give entirely consistent results during the 2 yr. Although DeKalb 1295 had the highest infection levels at all positions in 1978 and was very near the highest in 1980, Pioneer Brand UC5-2533 had the highest infection levels at all positions in 1980 but had less than average infection in 1978. The pinbar technique and to a certain extent the kernel injection technique showed Pioneer Brand 3030 and Dixie 55 to have relatively low infection levels, whereas DeKalb 1295 had a high level with both techniques.

We believe that pinbar inoculation shows the most potential of the techniques tested for determining the reaction of maize to kernel infection by *A. flavus*. This technique provided relatively high levels of infection with relative ease of inoculation and aquisition of kernels from ears. The inoculum delivered to kernels was only the amount that adhered to the pins, and this technique did not necessitate injection of a droplet of inoculum that might flow to adjacent kernels from the inoculated kernel. Furthermore, it was possible to obtain as many as 50–70 kernels of each row position for assay with each inoculation with the 100-mm pinbar. This number can easily be doubled by inoculating each ear twice, on opposite sides. This technique provides sufficient kernels from which data on aflatoxin contamination could be obtained in addition to percentage kernel infection. One disadvantage of the kernel injection technique was the time and care required to obtain and assay the relatively few kernels from each harvested ear.

With the pinbar inoculation technique, visible *A. flavus* growth was generally abundant on inoculated kernels, but very few kernels

adjacent to the inoculated row had visible *A. flavus* growth. In a study in Indiana, Rambo et al (16) reported similar results. Although they found 15% *A. flavus* infection in nearby kernels, they detected no aflatoxin in these kernels. The generally warmer Mississippi environment might favor more *A. flavus* growth. Phytotron studies (5,18) indicate that high day/night temperatures favor aflatoxin production and infection by *A. flavus*.

Interference competition by other fungi that inhabit maize kernels is known to reduce aflatoxin production by *A. flavus* (3,20), and these fungi could possibly inhibit growth by *A. flavus*. *Fusarium moniliforme* and *Cephalosporium acremonium* are especially common in maize kernels produced in Mississippi (6,7); it is possible that these fungi could significantly influence year-to-year variation in *A. flavus* infection or aflatoxin production, or both.

We were unable to detect a correlation between kernel hardness and susceptibility to *A. flavus* kernel infection. Hence, we feel it is safe to assume that kernel hardness is not a significant factor in resistance. Other factors such as pericarp thickness or toughness, pericarp breakage in relation to grain drying, or physiological factors associated with kernels or silks may be important in determining resistance to kernel infection. At present there is no clearly demonstrated resistance in maize to kernel infection by *A. flavus*.

LITERATURE CITED

1. Anderson, H. W., Nehring, E. W., and Wichser, W. R. 1975. Aflatoxin contamination of corn in the field. J. Agric. Food Chem. 23:775-782.
2. Christensen, C. M., and Kaufman, H. H. 1968. Grain Storage Fungi. University of Minnesota Press, Minneapolis. 153 pp.
3. Christensen, C. M., Nelson, G. H., Speers, G. M., and Mirocha, C. J. 1973. Results of feeding tests with ratios containing grain invaded by a mixture of naturally present fungi plus *Aspergillus flavus* NRRL 2999. Feedstuffs 45:20-21.
4. Fennell, D. I., Lillehoj, E. B., and Kwolek, W. F. 1975. *Aspergillus flavus* and other fungi associated with insect-damaged field corn. Cereal Chem. 52:314-321.
5. Jones, R. K., Duncan, H. E., Payne, G. A., and Leonard, K. J. 1980. Factors influencing infection by *Aspergillus flavus* in silk-inoculated corn. Plant Dis. 64:859-863.
6. King, S. B. 1981. Time of infection of maize kernels by *Fusarium moniliforme* and *Cephalosporium acremonium*. Phytopathology 71:796-799.
7. King, S. B., and Scott, G. E. 1981. Genotypic differences in maize to kernel infection by *Fusarium moniliforme*. Phytopathology 71:1245-1247.
8. Lancaster, M. C., Jenkins, F. P., and Phillip, J. M. 1961. Toxicity associated with certain samples of groundnuts. Nature 192:1095-1096.
9. LaPrade, J. C., and Manwiller, A. 1976. Aflatoxin production and fungal growth on single cross corn hybrids inoculated with *Aspergillus flavus*. Phytopathology 66:675-677.
10. LaPrade, J. C., and Manwiller, A. 1977. Relation of insect damage, vector, and hybrid reaction to aflatoxin B_1 recovery from field corn. Phytopathology 67:544-547.
11. Lillehoj, E. B., Kwolek, W. F., Manwiller, A., Durant, J. A., LaPrade, J. C., Horner, E. S., Reid, J., and Zuber, M. S. 1976. Aflatoxin production in several corn hybrids grown in South Carolina and Florida. Crop Sci. 16:483-485.
12. Lillehoj, E. B., Kwolek, W. F., Shannon, G. M., Shotwell, O. L., and Hesseltine, C. W. 1974. Aflatoxin occurrence in 1973 corn at harvest. I. A limited survey in the southeastern U.S. Cereal Chem. 52:603-611.
13. Lillehoj, E. B., Kwolek, W F., Vandergraft, E. E., Zuber, M. S., Calvert, O. H., Widstrom, N., Futrell, M. C., and Bockholt, A. J. 1975. Aflatoxin production in *Aspergillus flavus*-inoculated ears grown at diverse locations. Crop Sci. 15:267-270.
14. McMillian, W. W., Wilson, D. M., and Widstrom, N. W. 1978. Insect damage, *Aspergillus flavus* ear mold, and aflatoxin contamination in south Georgia corn fields in 1977. J. Environ. Qual. 7:564-566.
15. Nagarajan, V., and Bhat, R. V. 1972. Factor responsible for varietal differences in aflatoxin production in maize. J. Agric. Food Chem. 20:911-913.
16. Rambo, G. W., Tuite, J., and Crane, P. 1974. Preharvest inoculation and infection of dent corn ears with *Aspergillus flavus* and *A. parasiticus*. Phytopathology 64:797-800.
17. Taubenhaus, J. J. 1920. A study of the black and yellow molds of ear corn. Tex. Agric. Exp. Stn. Bull. 270:3-10.
18. Thompson, D. L., Lillehoj, E. B., Leonard, K. J., Kwolek, W. F., and Zuber, M. S. 1980. Aflatoxin concentration in corn as influenced by kernel development stage and postinoculation temperature in controlled environments. Crop Sci. 20:609-612.
19. Ullstrup, A. J. 1978. Corn diseases in the United States and their control. U.S. Dep. Agric.-ARS. Agric. Handb. 199. 55 pp.
20. Wicklow, D. T., Hesseltine, C. W., Shotwell, O. L., and Adams, G. L. 1980. Interference competition and aflatoxin levels in corn. Phytopathology 70:761-764.
21. Wilson, D. M., McMillian, W. W., and Widstrom, N. W. 1979. Field aflatoxin contamination of corn in south Georgia. J. Am. Oil Chem. Soc. 56:798-799.
22. Widstrom, N. W., Sparks, A. N., Lillehoj, E. B., and Kwolek, W. F. 1975. Aflatoxin production and lepidopteran insect injury on corn in Georgia. J. Econ. Entomol. 68:855-856.
23. Widstrom, N. W., Wiseman, B. R., McMillian, W. W., Kwolek, W. F., Lillehoj, E. B., Jellum, M. D., and Massey, J. H. 1978. Evaluation of commercial and experimental three-way corn hybrids for aflatoxin B_1 production potential. Agron. J. 70:986-988.
24. Zuber, M. S., Calvert, O. H., Kwolek, W. F., Lillehoj, E. B., and Kang, M. S. 1978. Aflatoxin B_1 production in an eight-line diallel of *Zea mays* infected with *Aspergillus flavus*. Phytopathology 68:1346-1349.
25. Zuber, M. S., and Lillehoj, E. B. 1979. Status of the aflatoxin problem in corn. J. Environ. Qual. 8:1-5.