Effect of Nitrogen Fertilizer, Planting Date, and Harvest Date on Aflatoxin Production in Corn Inoculated with *Aspergillus flavus*

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ABSTRACT

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Concentrations of aflatoxin B_1 were measured in artificially wounded and inoculated kernels of a short-season and a full-season corn cultivar grown in the Tidewater area of North Carolina, where incidence of aflatoxin from natural infection by *Aspergillus flavus* is low. In 1978, isolate 160 of *A. flavus* grew well but produced very little aflatoxin in inoculated ears; in 1979, it produced moderate levels while a second isolate, 3357, produced high levels. No significant difference was established between cultivars in either year, but corn planted 9 May 1979 had more aflatoxin than corn planted 11 April 1979. Significantly more aflatoxin was produced in grain harvested late (18% moisture) than in that harvested early (28% moisture). Aflatoxin B_1 concentrations were consistently higher in corn grown in plots low in nitrogen. These results suggest that corn planted and harvested late and produced under nitrogen stress is a better substrate for preharvest aflatoxin production than corn grown under good management practices and supplied with adequate nitrogen.

Aflatoxin B_1 is a carcinogenic, secondary metabolite produced by *Aspergillus flavus* Link ex Fries and *A. parasiticus* Speare in corn (*Zea mays* L.) kernels. Discovered in the 1960s (9), aflatoxins have become recognized as an economically significant source of preharvest (10,11,22) and postharvest (14,17) loss of grain quality, particularly in southern corn production areas. Current research has focused on weather conditions that affect the amount of preharvest aflatoxin in corn, including drought stress during the silking to late dough stage of grain development (6,23), rainfall during harvest periods (6,12), and high temperatures that favor the growth and development of *A. flavus* (7,12).

Surveys conducted in 1977 and 1978 in North Carolina indicated that high levels of aflatoxin in preharvest corn are negatively correlated with yield (2). These surveys and corroborating field experiments (6) also demonstrated that preharvest aflatoxin is less severe in the high-yielding Tidewater region of North Carolina than in corn produced on sandy coastal plain soils. Reduced capacity to hold water, inhibition of fertilizer uptake because of drought, and increased leaching of mineralized and supplemental nitrogen have been suggested as predisposing factors that increase aflatoxin contamination of field corn (5). Anderson et al (1) reported higher levels of aflatoxin in corn fertilized with 60 units of nitrogen than in corn fertilized with 140 units of nitrogen.

These experiments examined the influence of cultivar, planting date, and harvest date on aflatoxin production in artificially inoculated ears of corn grown in a region of North Carolina where aflatoxin contamination is traditionally low (2). Results of 1978 experiments led us to expand the 1979 tests to include the variables of nitrogen fertilization and an additional isolate of *A. flavus* that produces high levels of aflatoxin.

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MATERIALS AND METHODS

Experimental design. In 1978 and 1979, experimental plots were planted at the Tidewater Research Station near Plymouth, NC, on a Portsmouth soil containing about 6% organic matter. The 1978 experiment was a $2^2 \times 3$ factorial, randomized complete block design with six replications. Seed of Pioneer Brand (PB) cultivars 3780 (short season), 3368A (midseason), and 3147 (full season) were planted on 3 April and 3 May 1978. Subplots consisted of eight rows 12.2 m long and 0.91 m apart. Two rows of each subplot were inoculated and subsequently harvested on one of two harvest dates. The corn was harvested at about 28% moisture on the early harvest date and 18% on the later date. A total of 72 samples was examined.

The 1979 experiment was a split plot, $2^3 \times 3$ factorial, randomized complete block design with four replications. Liquid 2-6-12 (N-P-K) was broadcast at 560 kg/ha over the entire field before planting. The split plot compared the effects of nitrogen at two application rates. The low rate was 11.2 kg/ha, or that obtained only from the preplant application. The higher rate was 145.7 kg/ha, obtained by adding NH_4NO_3 to the soil surface 30 days after planting. Previous work has shown that the average yield of a midseason cultivar on similar Portsmouth soils is approximately 4,400 kg/ha with no supplemental nitrogen (8).

Cultivars PB 3780 and PB 3147 were planted in the subplots (four rows 10.2 m long) 11 April and 9 May 1979. The subplots were divided into thirds and randomly assigned to one of three inoculum treatments, which were two isolates of *A. flavus* and a water check. Two rows of each four-row subplot were randomly assigned to early or late harvest. A total of 192 samples was examined.

Isolate. Two isolates of *A. flavus* were used. Isolate 160 was obtained from corn and maintained on Sabouraud dextrose agar. This isolate produces aflatoxin when grown on sterilized rice and was routinely used for aflatoxin B_1 production for dose studies in the Poultry Science Department, North Carolina State University. Isolate 3357 was obtained from K. J. Leonard (Department of Plant Pathology at the university) as a subculture of NRRL-3357 obtained from E. B. Lillehoj (Southern Regional Research Center, New Orleans, LA 70179). These isolates produce large quantities of aflatoxin and have been used successfully in field studies (7,12,21).

The isolates were grown for 10 days on Czapek-Dox agar at 25 C. Conidia were harvested in sterile distilled water containing 0.01% Titron X-100. The concentration of the conidial suspensions was adjusted to 10^6 conidia per milliliter.

Inoculation procedure. Plants were inoculated as described by Zuber et al (21). About 21 days after half of the silk had emerged, husks were pulled back and two rows of kernels were injured with an

acrylic pinboard embedded with a single row of 15 stainless steel needle points (3 mm long and 4 mm apart). The injured area was then sprayed with 0.5 ml of inoculum. Husks were put back in place and secured with a rubber band. A cloth drawstring bag (25 × 15 cm) was placed over the ear and secured to exclude insects. Larvae of the corn earworm (*Heliothis zea* Boddie) were removed from the silk channel at the time of inoculation if they were present.

Tissue analysis. In the 1979 experiment, we monitored the nutrient status of plants in each subplot of planting date × cultivar. The leaf just above the dominant ear leaf was removed from four randomly selected plants beginning 18 June and continuing until senescence. Leaves were placed in plastic bags on ice or in a cold room at 2 C until nutrient analysis could be performed (maximum delay, 72 hr). The nutrient status of maturing grain was determined in a similar manner with four randomly selected, dominant ears that were harvested, dried, and ground before analysis. The percentage of nitrogen was determined by the Kjeldahl procedure at the Plant Analysis Laboratory of the North Carolina Department of Agriculture.

Harvesting and aflatoxin analysis. Ears were hand harvested and machine shelled; kernels adhering to the cob were removed by hand and included in the sample. Samples were coarse ground, riffle divided, finely ground to pass through a 20-mesh screen, and dried in a forced-air oven at 70 C for 78 hr as previously described (6).

Samples were extracted for aflatoxins using a modified Pons procedure (16). Activated thin-layer chromatograms (Eastman 6061, Eastman Kodak Company, Rochester, NY 14650) were spotted with 1 μl of an appropriately diluted extract and developed in an unsaturated chamber containing a 90:5:5 (v/v) solution of benzene:methanol:acetic acid. Aflatoxin B_1 concentrations were estimated visually under ultraviolet light (366 nm) by comparison with commercially prepared standards (Applied Science Laboratory, State College, PA 16801). The presence of aflatoxins was confirmed in representative samples with dilute sulfuric acid (18).

RESULTS AND DISCUSSION

In 1978, low levels of aflatoxin B_1 were detected in three of 72 samples inoculated with isolate 160. The aflatoxin was found at 30 μg/g in two samples of PB 3368A planted in April and harvested at 18% moisture and at 10 μg/g in one sample of PB 3147 planted in May and harvested at 28% moisture. Contamination was low despite the fact that visible growth and sporulation of *A. flavus* were found in the inoculated areas of 96% of the ears harvested. The ability of isolate 160 to produce aflatoxin in corn was reconfirmed by inoculating ears of Gaspe #g 3740 'Gaspe flint,' which was grown in the Southeastern Regional Controlled Environment Laboratory (Phytotron, North Carolina State University). This cultivar was selected because of its short generation time and dwarf growth habit. Five ears of Gaspe #g 2740 were inoculated about 21 days after silk emergence. Ears were harvested 3 wk later, and the kernels from each ear were bulked and analyzed for aflatoxin. All five samples contained aflatoxin B_1, with an average of 450 μg/kg per sample.

Surveys and experimental work conducted in North Carolina demonstrated that aflatoxin B_1 contamination of field corn was correlated with several factors, including drought stress (particularly from silking to the late dough stage) and reduced yields (2,6). Drought stress and low yields are not common on the richly organic soils of the Tidewater region (19). The average yield in the 1978 experiment was 7,757 kg/ha (123.6 bu/acre), which is well above the state average but comparable with average Tidewater yields (8).

Drought stress has been suggested as a factor predisposing corn to invasion by *A. flavus* and production of aflatoxin B_1 (6). It also alters the uptake and translocation of nitrogen in corn (20). Naik et al (15) demonstrated that the protein fraction of groundnuts contributes to the higher levels of aflatoxin synthesis by *A. flavus*. They also observed that certain amino acids, particularly proline, support higher levels of aflatoxin synthesis by *A. flavus* when incorporated as the nitrogen source in a defined medium. Higher levels of proline have been found in corn grown under drought (4) and nitrogen stress (3).

We concluded that the mineralization of nitrogen on these soils, which are rich in organic matter, and the high rates of fertilization in the Tidewater region may

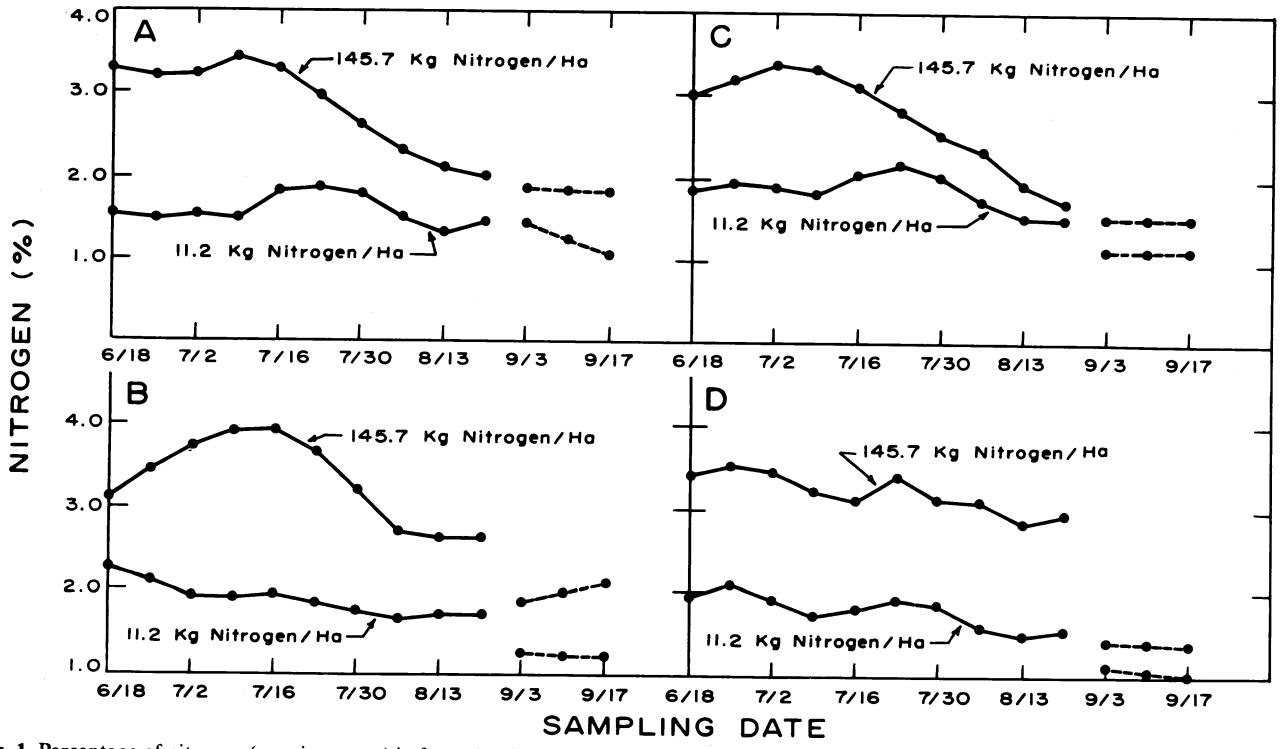

Fig. 1. Percentage of nitrogen (running mean) in four planting date × cultivar subplots. **(A)** Pioneer Brand (PB) 3780 planted 11 April. **(B)** PB 3780 planted 9 May. **(C)** PB 3147 planted 11 April. **(D)** PB 3147 planted 9 May. Tissue analyzed by Kjeldahl method from plots receiving nitrogen at 145.7 kg/ha and at 11.2 kg/ha. Leaves (solid line) and grain (dashed line) were sampled weekly during the growing season.

Table 1. Effect of *Aspergillus flavus* isolates, planting dates, and harvest dates on aflatoxin B_1 concentrations in corn plots receiving different rates of nitrogen

Inoculum	Planting date	Time of harvest[a]	Aflatoxin B_1 (μg/kg)[b] Nitrogen at 145.7 kg/ha	Nitrogen at 11.2 kg/ha
Isolate 160	April 11	1	19	64
		2	63	74
	May 9	1	37	146
		2	63	194
Isolate 3357	April 11	1	882	1,950
		2	1,525	3,875
	May 9	1	2,000	4,375
		2	1,963	4,875
Water check	April 11	1	115	371
		2	204	609
	May 9	1	344	694
		2	482	1,050

[a] Time of harvest was based on moisture content: 1 = 26–28%, 2 = 16–18%.
[b] Values represent the arithmetic mean of four replicates of PB 3780 and PB 3147.

Table 2. Analysis of variance[a] for aflatoxin B_1 (log B_1 + 1) in inoculated corn from plots fertilized with two rates of nitrogen

Source	d.f.	Mean square High rate of N[b]	Mean square Low rate of N[c]	$P > F$ High rate of N[b]	$P > F$ Low rate of N[c]
Planting date	1	31.2	16.8	0.067	0.003**
Replicate × planting date	3	3.9	0.2		
Cultivar	1	3.5	0.4	0.163	0.57
Replicate × cultivar	3	1.0	1.0		
Cultivar × planting date	1	5.7	0.01	0.159	0.9
Replicate × cultivar × planting date	3	1.6	0.67		
Isolate	2	130.1	107.6	0.001**	0.0001**
Replicate × isolate × cultivar × planting date	24	1.9	0.5		
Harvest date	1	8.1	1.4	0.013**	0.04*
Replicate × isolate × harvest date × cultivar × planting date	24	1.1	0.3		

[a] * = significant at $P = 0.05$, ** = significant at $P = 0.01$.
[b] High rate of nitrogen = 145.7 kg/ha.
[c] Low rate of nitrogen = 11.2 kg/ha.

yield corn that is a poor substrate for aflatoxin production by isolate 160 of *A. flavus*. We thus decided to include nitrogen fertilization as a variable in the 1979 experiments. The percentage of nitrogen (running mean) during the sampling period for each planting date × cultivar subplot is presented in Figure 1.

In 1979, isolate 160 produced moderate levels and isolate 3357 produced high levels of aflatoxin B_1 (Table 1). Aflatoxin B1 was frequently detected in treatments sprayed only with sterile distilled water. The ears probably became infected during the inoculation procedure, because visible growth of *A. flavus* was observed in some ears of these treatments at the time of harvest. They may have been infected with isolate 3357, because they contained more aflatoxin than ears inoculated with isolate 160. Analysis of variance (Table 2) revealed a significant effect of isolate ($P = 0.001$).

Grain harvested at 28% moisture contained significantly less aflatoxin B_1 than grain harvested at 18% moisture ($P = 0.01$). Planting date (April vs. May) had a significant effect within the low-nitrogen plots ($P = 0.003$) and just missed significance within the high-nitrogen plots ($P = 0.067$). Cultivars did not have a significant effect.

Jones et al (6) reported significantly less aflatoxin B_1 from natural infection in April plantings in corn vs. May plantings. They also found less aflatoxin in a short-season cultivar (PB 3780) planted in April than in a full-season one (PB 3147) similarly planted. They suggested that lower levels of airborne inoculum during the silking period of PB 3780 planted in April may have contributed to this difference. We found no significant difference between cultivars in aflatoxin B_1 level within planting dates when ears were wounded and inoculated. This result supports the claim that inoculum availability is the limiting factor under natural conditions. The observed difference in aflatoxin concentration in corn planted on different dates in the current study and in work reported earlier (6) involving natural infection suggests that factors other than inoculum availability are contributing to higher levels of aflatoxin B_1 in corn planted in May rather than April.

In 1979, regardless of the isolate used, less aflatoxin B_1 was detected in treatments receiving the high rate of nitrogen (145.7 kg/ha) than in treatments receiving the low rate (11.2 kg/ha). Low-nitrogen plots had 2.4 times more aflatoxin B_1 than high-nitrogen plots when results were averaged across cultivar, planting date, and isolate. This supports the reports of Anderson et al (1) and Lillehoj and Zuber (13) that plant stress associated with reduced fertilization increases the incidence of aflatoxin contamination. Our experiments did not expose corn to drought stress; consequently, the different nitrogen levels in the high- and low-nitrogen plots can be attributed to the amount of actual nitrogen applied. Although nitrogen levels were not subjected to statistical analysis because the fertilizer treatments were not randomized, we found consistently higher levels of total nitrogen in leaf and grain tissues and lower levels of aflatoxin B_1 in plots that received 145.7 kg/ha of nitrogen rather than the low rate of 11.2 kg/ha. We suggest that inadequate nitrogen fertilization alters the nutritional status of preharvest corn and makes it a better substrate for aflatoxin production. We believe that this area could be further explored.

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