Pathogenesis in Aspergillus Ear Rot of Maize: Aflatoxin B_1 Levels in Grains Around Wound-Inoculation Sites

M. G. Smart, O. L. Shotwell, and R. W. Caldwell

First and second authors: Fermentation Biochemistry Research Unit and Mycotoxin Research Unit, USDA-ARS, Northern Regional Research Center, 1815 North University Street, Peoria, IL 61604.

Third author: Department of Plant Pathology, Russell Laboratories, 1620 Linden Drive, University of Wisconsin, Madison, WI 53706.

The mention of firm names or trade products does not imply endorsement or recommendation by the U.S. Department of Agriculture over other firms or similar products not mentioned.

We thank Dr. E. B. Smalley (University of Wisconsin, Madison) for use of the Biotron facility. Dr. D. T. Wicklow provided stimulating discussion of the work, for which we are grateful. We also thank Dr. T. Nelsen (USDA-ARS, Peoria, IL) for statistical advice. One of us (M. G. S.) recognizes his debt to the kidney donor.

Accepted for publication 21 February 1990.

ABSTRACT

Smart, M. G., Shotwell, O. L., and Caldwell, R. W. 1990. Pathogenesis in aspergillus ear rot of maize: Aflatoxin B_1 levels in grains around wound-inoculation sites. Phytopathology 80:1283-1286.

Aflatoxin contamination of preharvest maize intermittently presents serious problems in grain storage and in animal health. To determine whether this mycotoxin can be translocated through the ear in the absence of hyphae, we harvested 21 grains from around each of 21 wound-inoculation sites of maize ears matured at 34/30 C (day/night). Individual grains were analyzed for aflatoxin by enzyme-linked immunosorbent assay. Maize spikelets are borne in pairs and, if aflatoxin is transported in the rachis, grains in a pair should have similar toxin levels, but nonpaired grains may or may not. Statistical treatment showed that no two grains chosen at random had different average toxin levels: there was no pattern discernible in toxin accumulation. Highly contaminated individual grains rarely had highly contaminated neighbors. Finally, of the 413 grains assayed, almost 80% either were aflatoxin-positive and showed signs of the fungus or were not aflatoxin-positive and had no signs of the fungus. Only 58 grains (14%) had detectable toxin levels without fungal signs. We conclude that long-distance transport of aflatoxin does not occur in infected ears independently of the hyphae.

Additional keywords: Aspergillus flavus, ELISA, mycotoxins, *Zea mays.*

Accumulated aflatoxin in stored grain and seeds is concentrated in a small number of individuals (2,7,12,13). Lee et al (8) reported a pattern of highly variable toxin levels in single maize grains taken from the *Aspergillus flavus* Link:Fr. infested portion of three insect-damaged ears. They also found highly contaminated grains (up to 8×10^4 ng aflatoxin g^{-1}) adjacent to grains lacking detectable aflatoxin. The pattern of aflatoxin accumulation in individual grains around an inoculation site has not been investigated directly. However, clues to toxin variability are found in the data of Wicklow and coworkers (18,19), who grouped intact grains surrounding sites of inoculation with *A. flavus* according to their proximity to the wound. The authors found that, on average, aflatoxin levels decreased with increasing distance from the wound, but, within groups, levels were variable.

Very little is known of the mechanics of toxin accumulation in seeds on developing maize ears. In the most general sense, the variability evident in work published previously could have arisen in two ways: either the toxin alone (in the absence of hyphae) was able to move by diffusion through tissues of the rachis to contaminate other grains, or the grains first were infected by hyphae before aflatoxin could accumulate. The data of Lee et al (8) are inconclusive on this point but did identify grains which had aflatoxin but no visible evidence of fungal colonization. In this regard, Mertz et al (10) injected aflatoxin into an internode of the ear shank and recovered aflatoxin from the grains 1 mo later, indicating toxin movement.

We undertook this study to further document the variability in toxin levels and to investigate the mechanism of toxin accumulation in grain. We harvested 21 grains from around each of 21 wound-inoculation sites and determined aflatoxin levels in the individual grains. We also noted the incidence of fungal signs in the grains. The data provide clues to the mechanics of pathogenesis in this disease and form the basis of a histological study of the in situ growth of *A. flavus* (14).

MATERIALS AND METHODS

Suscept and pathogen material. Maize (*Zea mays* L. DeKalb hybrid XL12) was grown under controlled conditions at the University of Wisconsin Biotron, Madison (1). Briefly, the plants were grown under a 12-hr/12-hr day/night photoperiod with a daytime flux of 600 $\mu Em^{-2}s^{-1}$ photosynthetically active radiation measuring 1.5 m above the topmost leaf). The temperature was 30 C during the day and 20 C during the night until pollination was complete. After pollination, the temperature was raised to 34 C during the day and 30 C during the night. Relative humidity was approximately 85%, and the plants were watered liberally to prevent stress. The inoculum used was a mixture of conidia from 10 isolates of *A. flavus* obtained from the collection of the Northern Regional Research Center. Their numbers were as follows: NRRL 6536, NRRL 6537, NRRL 6539, NRRL 6540, NRRL 6576, NRRL 6577, NRRL 6578, NRRL 6579, NRRL 6580, and NRRL 6581. All isolates were collected originally from maize (17). A 10-isolate mixture was used because a single isolate did not spread from the wound sites under a 30/20 day/night temperature regime (see the accompanying paper (14) for a full discussion of this point).

Preparation of inoculum and inoculation. The isolates were grown separately on malt extract agar for 7 days at 22 C in the dark. Conidial suspensions were prepared by flooding the plates with sterile, distilled water containing 0.01% Triton X-100. Spore concentration was adjusted to 1×10^6 ml^{-1}, and equal volumes of the 10 suspensions were combined. Maize ears were wounded at 21 days after silk emergence by inserting a sterile toothpick through the husks into a grain and the underlying rachis. The toothpick was withdrawn, dipped in the spore suspension,

and replaced in the wound. Four ears were treated in this way, each ear inoculated six times in two vertical columns of three wounds, columns being on opposite sides of the ear. At maturity, approximately 28 days postinoculation under these conditions (average kernel moisture was 15–16%), whole ears were harvested and shipped to Peoria for analysis. Ears were stored at 4 C for a maximum of 10 days before removal of tissues for analysis. No further fungal development occurred during this time.

Analysis: microscopy and aflatoxin determination. Twenty-one grains were removed in a mosaic pattern from around each of 21 wound sites in the four ears (Fig. 1). Three wounds of the original 24 were contaminated with *Aspergillus niger* Tiegh. and discarded. Not all grains were present in every mosaic: unfertilized ovaries were obliterated by their developing neighbors in a small number of instances. Grains next to the wounded spikelet were examined for damage by the toothpick. Any such damaged grains (there were few) were not collected. The least number of replicates of a grain position was 19 (for grains designated −d, see Fig. 1).

The test grains were separated from the adherent proximal rachilla ("tip cap") and bracts, and then halved longitudinally so as to include one-half of the embryo in each piece. One-half of each grain was dried at 90 C for 1 hr, capped tightly in a vial, and refrigerated (4 C) until aflatoxin was determined. The other half of each grain was fixed and stored at 4 C in 8% w/v paraformaldehyde in 0.025 M phosphate buffer, pH 6.8. All of these latter half-grains (there were 413) were examined for signs of the fungus at ×400 with a stereomicroscope. Signs observed were sporulation, sclerotium formation, and vegetative hyphae.

The corresponding grain halves were assayed for aflatoxin (measured as aflatoxin B_1) by an enzyme-linked immunosorbent assay (ELISA, Agritech Systems, Inc., Portland, ME). The technique was modified for use on single half-grains: Each sample was weighed and then soaked overnight in 5.0 ml of extraction medium (70% methanol, 29% water, and 1% dimethylformamide, all v/v). Soaked grains were comminuted in the medium and left a further 4 hr at 22 C. The particulates were removed by filtration through glass fiber, and a 15-μl aliquot of the filtrate was diluted and processed for ELISA according to the manufacturer's instructions. Aflatoxin was calculated as ng g^{-1}, and the minimum detectable level was approximately 10 ng g^{-1} for a 0.1-g half-grain. Because each grain position was represented at least 19 times, the aflatoxin level for each particular grain was determined just once. The values reported for individual half-grains are subject to a variability of plus or minus 10%, based upon multiple tests of several half-grains.

RESULTS

The averages, medians, and ranges of aflatoxin B_1 levels for each grain position are shown in Table 1. The average values varied from 562 ng g^{-1} for the 19 grains positioned at −d to 9,220 ng g^{-1} for the 20 grains positioned at b (see Fig. 1). Despite this disparity, statistical treatment of the means by the general linear model procedure showed no difference between the average toxin levels of any two grains chosen at random ($P = 0.05$). The extreme variation in toxin levels within a particular grain position accounted for the large standard deviations to the means and was illustrated by the median toxin levels and associated ranges. No toxin was detected in many grains, which gave median levels much less than the mean toxin levels. The upper limit of aflatoxin levels in an individual grain was also extremely variable: one grain at position −d had about 2,500 ng g^{-1} aflatoxin while the most heavily contaminated grain at position −f had over 86,000 ng g^{-1} aflatoxin.

Naturally, these extremes in toxin variability within a grain position lead to similar extremes of grain-to-grain variability. In Table 2, each of the 25 most heavily contaminated individual grains is identified along with its aflatoxin level. The toxin concentrations of the two grains laterally adjacent (that is, in the same row in Fig. 1) are given also. It was rare to find that either of the two flanking grains shared a high toxin level with the heavily contaminated grain. Only four such cases occurred (bracketed in the table); three were in ear four. Indeed, two of these cases were in wound 2 of ear 4 (grain 2 with 3 and grain −b with −c). Both neighbors of two highly contaminated grains had no detectable aflatoxin (ear 2, wound 2, grain −c; and ear 2, wound 6, grain b). The wounded grain identified in the table had a surprisingly low aflatoxin level and, in fact, no wounded grain had more than 1,000 ng g^{-1} aflatoxin. In eight wounds, no aflatoxin at all was detected, giving a median value of 18 ng^{-1}. All of the wounded kernels were rotted and consisted only of pericarp remnants, hyphae, and sclerotia.

When the 413 grains were examined microscopically, 248 (60%) showed signs of *A. flavus*. Of these, 221 (53.5% of all grains) were also contaminated with toxin. Of the 165 grains without signs of the fungus, 107 (26% of all grains) had no detectable aflatoxin. Only 58 grains (14% of all grains) had detectable toxin levels without obvious fungal signs.

DISCUSSION

Maize grains are borne singly on spikelets, but the spikelets themselves are arranged in pairs (6,9,16). The vascular systems of any particular spikelet pair converge at their insertion into one of the major vascular bundles serving the whole ear (16). In Figure 1, if the wounded grain (w) and grain 1 had been a spikelet pair, then 2, 3; a, b; −g, −h; etc., would have been pairs also. Conversely, if grain w and grain −1 had been a pair, then b, c; −b, −c; etc., would have been pairs.

		e	f	g	h
		a	b	c	d
−2	−1	w+	1	2	3
−d	−c	−b	−a		
−h	−g	−f	−e		

Fig. 1. Pattern of contiguous maize caryopses, removed from 21 wound-inoculation sites of four *Aspergillus flavus*-infested ears. The tip and butt of the ear are to the top and bottom of the figure, respectively. Thus, grains e, a, w, −b, and −f are considered to be in columns while grains a, b, c, d, and −2, −1, w, 1, 2, and 3 are in rows. "Laterally adjacent" grains are within rows (e.g., grain 2 is laterally adjacent to grains 1 and 3). + is the wounded grain at the inoculation site.

TABLE 1. Aflatoxin B_1 values measured by enzyme-linked immunosorbent assay for each grain harvested in a pattern around 21 wound-inoculation sites in maize

Grain position[a]	Toxin concentration (ng g^{-1})		
	Average ± SD[b]	Median	Range
1	4,103 ± 7,632	1,956	ND[c]-31,145
2	4,189 ± 9,996	864	ND-39,026
3	2,899 ± 6,036	850	ND-27,823
a	2,236 ± 3,896	575	ND-16,984
b	9,220 ± 19,830	321	ND-81,736
c	1,471 ± 3,112	17	ND-13,320
d	3,932 ± 8,809	531	ND-39,429
e	2,997 ± 5,529	1,525	ND-24,602
f	1,305 ± 1,680	374	ND- 5,415
g	687 ± 941	213	ND- 3,342
h	1,898 ± 6,057	75	ND-27,943
−1	3,886 ± 8,494	1,030	ND-28,706
−2	4,178 ± 9,800	142	ND-37,817
−a	2,115 ± 3,885	450	ND-17,031
−b	4,440 ± 12,948	437	ND-57,978
−c	6,110 ± 19,044	62	ND-84,180
−d	562 ± 902	ND	ND- 2,494
−e	3,990 ± 8,057	316	ND-25,721
−f	7,608 ± 21,139	106	ND-86,413
−g	1,709 ± 2,445	283	ND- 9,435
−h	1,725 ± 3,273	339	ND- 2,860

[a] See figure 1.
[b] These means were compared two at a time by the general linear model procedure. No two means chosen at random differ significantly ($P = 0.05$).
[c] ND = None detected.

This anatomical arrangement has important consequences in the context of toxin movement. The data of Mertz et al (10) imply that it is possible for toxin to move in the ear, at least in uninfected maize. If toxin can also move independently of the hyphae in infected ears, it must do so through the vascular system because the extreme grain-to-grain variability of aflatoxin levels (Table 2) precludes simple diffusion. Such movement in the vascular system would lead to a distinct pattern of toxin accumulation. The spikelet pairs in the same column (see Fig. 1) as the wounded grain should have decreasing toxin levels from pair to pair as one samples further from the toxin source (initially the wound). Within the spikelet pairs, however, toxin levels should be the same because their vascular systems emerge from the main vascular bundles at almost the same point. Columns of spikelet pairs other than the one containing the wound-inoculation site should have different (initially lower) toxin levels in the grains since vascular connections between major bundles are not extensive (16); but, again, toxin levels within spikelet pairs should be the same.

Our data supply three reasons for rejecting the hypothesis that aflatoxin can move in the rachis independently of hyphae. First, statistical treatment of the average toxin values at each position showed no difference between any two compared at random (except as could be expected by chance alone), that is, there was no pattern evident in the accumulation of aflatoxin. Even though this implies that the members of spikelet pairs had the same aflatoxin level, the lack of any discernible pattern overall argues against independent toxin movement.

The variability evident at the level of individual grains in the raw data (of which Table 2 is a small sample) also argues against independent diffusion of toxin in the rachis vascular system. The data of the table show that individual pairs of grains with similar toxin levels (meaning within 50% of each other) were quite uncommon: there were just four. Two of these pairs were in ear four, wound two (grain 2 with 3, grain −b with −c) and could not simultaneously have belonged to spikelet pairs (see Fig. 1). Six groups of the sampled neighbors (that is, groups of the two grains on either side of the highly contaminated grain) had toxin levels less than 20% of the particular value, and the four grains in two of these groups had no detectable aflatoxin.

Finally, the correlation between the presence of detectable toxin together with fungal signs, or the simultaneous absence of both, is quite good. Almost 80% of all grains fell into one of these categories. Only 58 grains (14% of the total) had detectable toxin, but no fungal signs. The median toxin level in this group was 392 ng g^{-1}. Most of these grains could have had superficial contamination from hyphae growing on the pericarp surface as, overall, this was relatively common. For the rest, including two very highly contaminated grains, there may have been short-distance (less than 250 μm) diffusion of toxin from hyphae in or on the proximal rachilla and floral axis ("tip cap") which was not sampled. Pea seeds, infected with *A. niger*, have shown the presence of a toxin in the embryo in the absence of hyphae (4). For these three reasons, we conclude that toxin accumulation in a grain is highly dependent upon the close proximity of the fungus and that long-distance transport of aflatoxin in the rachis does not occur.

We cannot close without commenting on the low toxin concentrations of the wounded grains. Such values could have arisen in three ways: toxin was transported from the wounds, no toxin was synthesized in the wounds, or toxin was destroyed. Toxin transport, at least over long distances in the rachis, did not occur. Previous experiments in this laboratory (Smart, Shotwell, and Elam, *unpublished*) showed toxin levels in excess of 8×10^4 ng g^{-1} in the wound of inoculated maize grown and matured at 30/20 C day/night. (Their neighboring grains, significantly, had very low [less than 50 ng g^{-1}] or undetectable aflatoxin levels.) The high toxin values in the wounds indicated synthesis at this lower temperature, in agreement with the results of other workers (see 15,18,19). Thus, we are left with the possibility that, at higher temperatures, aflatoxin was being destroyed. Aflatoxin disappeared from in vitro, axenic cultures of *Aspergillus*

TABLE 2. Aflatoxin concentrations in the 25 most heavily contaminated grains and their laterally adjacent neighbors. Grain toxin values greater than 15,000 ng g^{-1} are presented[a]

Position of center grain			Aflatoxin concentration (ng g^{-1})		
Ear	Wound position	Grain position	Center grain	Laterally adjacent grains	
1	2	b	81,736	539	1,078
1	3	−f	19,322	3,455	960
1	4	−a	17,031	187	...[c]
1	4	−e	23,476	86,413	...
1	4	−f	86,413	9,435	23,476
2	2	b	31,398	5,022	ND
2	2	−c	30,136	ND[b]	ND
2	2	−e	17,123	11	...
2	4	−1	28,706	3,441	ND[d]
2	5	e	24,602	...	991
2	6	b	17,607	ND	ND
3	5	h	27,943	12	...
3	5	−2	37,817	...	ND
3	6	1	31,145	78[d]	39,026
3	6	2	[39,026	31,145][e]	129
3	6	−1	28,238	3,086	78[d]
4	2	2	27,871	546	27,823
4	2	3	[27,823	27,871]	...
4	2	−2	26,610	...	1,971
4	2	−b	[57,978	84,180]	17
4	2	−c	84,180	1,166	57,978
4	2	−f	48,004	283	266
4	4	d	39,429	972	...
4	4	a	16,984	...	32,545
4	4	b	[32,545	16,984]	972

[a] Cf Figure 1.
[b] None detected.
[c] Grain outside sampled area.
[d] Wounded grain.
[e] Members of a grain pair, both of which have aflatoxin concentrations greater than 15,000 ng g^{-1}.

parasiticus Speare upon aging (3,5). But Payne et al (11) do not believe that the host is responsible for toxin degradation in planta. If toxin degradation did occur, as our evidence would suggest, it would also explain 15 badly rotted grains in the mosaics which had no detectable aflatoxin.

LITERATURE CITED

1. Caldwell, R. W., Smalley, E. B., and Hesseltine, C. W. 1984. Controlled environments to study mycotoxin production on maize. Pages 61-71 in: Toxigenic fungi–their toxins and health hazards. H. Kurata and T. Vero, eds. Elsevier, The Netherlands. 363 pp.
2. Cucullu, A. F., Lee, L. S., Mayne, R. M., and Goldblatt, L. A. 1966. Determination of aflatoxins in individual peanuts and peanut sections. J. Am. Oil Chem. Soc. 43:89-92.
3. Doyle, M. P., and Marth, E. H. 1978. Aflatoxin is degraded by heated and unheated mycelia, filtrates of homogenized mycelia and filtrates of broth cultures of *Aspergillus parasiticus*. Mycopathologia 64:59-62.
4. Harman, G. E., and Nash, G. 1972. Deterioration of stored pea seed by *Aspergillus ruber*: Evidence for involvement of a toxin. Phytopathology 62:209-212.
5. Huynh, V. L., and Lloyd, A. B. 1984. Synthesis and degradation of aflatoxins by *Aspergillus parasiticus* 1. Synthesis of aflatoxin B_1 by young mycelium and its subsequent degradation by aging mycelium. Aust. J. Biol. Sci. 37:37-43.
6. Kisselbach, T. A. 1949. The structure and reproduction of corn. Neb. Agric. Exp. Stn. Res. Bull. 161.
7. Lee, L. S., and Cucullu, A. F. 1978. Aflatoxin negative cottonseed in bright, greenish yellow fluorescent seed locks. J. Am. Oil Chem. Soc. 55:591.
8. Lee, L. S., Lillehoj, E. B., and Kwolek, W. F. 1980. Aflatoxin distribution in individual corn kernels from intact ears. Cereal Chem. 57:340-343.
9. Manns, T. F., and Adams, J. F. 1923. Parasitic fungi internal of seed corn. J. Agric. Res. 23:495-524.
10. Mertz, D., Lee, D., Zuber, M., and Lillehoj, E. 1980. Uptake and metabolism of aflatoxin by *Zea mays*. J. Agric. Food Chem. 28:963-966.
11. Payne, G. A., Hagler, Jr., W. M., and Adkins, C. R. 1988. Aflatoxin accumulation in inoculated ears of field-grown maize. Plant Dis. 72:422-424.
12. Shotwell, O. L., Goulden, M. L., and Hesseltine, C. W. 1974. Aflatoxin: Distribution in contaminated corn. Cereal Chem. 51:492-499.
13. Shotwell, O. L., Goulden, M. L., Lillehoj, E. B., Kwolek, W. F., and Hesseltine, C. W. 1977. Aflatoxin occurrence in 1973 corn at harvest. III. Aflatoxin distribution in contaminated, insect-damaged corn. Cereal Chem. 54:620-626.
14. Smart, M. G., Wicklow, D. T., and Caldwell, R. W. 1990. Pathogenesis in Aspergillus ear rot of maize: Light microscopy of fungal spread from wounds. Phytopathology 80:1287-1294.
15. Thompson, D. L., Payne, G. A., Lillehoj, E. B., and Zuber, M. S. 1983. Early appearance of aflatoxin in developing corn kernels after inoculation with *Aspergillus flavus*. Plant Dis. 67:1321-1322.
16. Weatherwax, P. 1916. Morphology of the flowers of *Zea mays*. Bull. Torrey Bot. Club 43:127-144.
17. Wicklow, D. T., and Horn, B. W. 1984. *Aspergillus flavus* sclerotia found in wound-inoculated prehavest corn. Mycologia 76:503-505.
18. Wicklow, D. T., Horn, B. W., Shotwell, O. L., Hesseltine, C. W., and Caldwell, R. W. 1988. Fungal interference with *Aspergillus flavus* infection and aflatoxin contamination of maize grown in a controlled environment. Phytopathology 78:68-74.
19. Wicklow, D. T., Horn, B. W., and Shotwell, O. L. 1988. Aflatoxin formation in preharvest maize ears coinoculated with *Aspergillus flavus* and *Aspergillus niger*. Mycologia 79:679-682.