Production of Aflatoxin in Wounded and Whole Maize Kernels by *Aspergillus flavus*

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ABSTRACT

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Wounded and nonwounded kernels of four inbred lines of maize were inoculated with *Aspergillus flavus* and were washed or not washed. The kernels and wash water were analyzed for aflatoxin B_1 (AFB_1) content. Inbred lines N141 and Mo17Ht had the highest levels of AFB_1 for the nonwashed, wounded treatment, whereas inbred lines Oh3A and Mo20W had the lowest. The nonwashed, wounded treatment yielded significantly more aflatoxin than other treatments, and the washed, whole kernels produced the least AFB_1. AFB_1 from the wounded series external mycelium was the highest for inbred N141 (1,238 ppb). For the whole-kernel series, the external contamination was also greatest for N141 (1,059 ppb). AFB_1 in the wash water from N141 and Mo17Ht was significantly greater than from the liquid from Mo20W and Oh3A. Aflatoxin content of the wash water from the kernel surface was low, indicating that the toxin was probably lost in the evaporation of the wash water and was not recovered in the analytical process.

Decapping mature maize kernels (*Zea mays* L.) is a useful tool for challenging genotypes in vitro with *Aspergillus flavus* Link ex Fries with the ultimate objective of finding fungal tolerance as well as minimal levels of aflatoxin B_1 (AFB_1). This laboratory method has been used for evaluating the responses of kernels to fungal growth and for measuring subsequent levels of aflatoxin among various maize genotypes (2–6,11,12). However, wounding kernels destroys any possible morphological types of resistance associated with the pericarp and the aleurone layer that may deter the entry of the pathogen.

The first objective of this study was to determine if whole kernels challenged with *A. flavus* would produce sufficient levels of AFB_1 to identify differences in genotypes, thereby negating the necessity of wounding the kernels. The second objective was to determine if the external conidia and mycelia on the kernels play a significant role in AFB_1 content of the kernels, and if so, to determine if greater levels are produced on the surface or the interior of wounded or whole kernels of different maize inbred lines. Some question has arisen among investigators concerned with AFB_1 as to whether or not the principal source of the toxin is on the surface of the maize kernels.

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MATERIALS AND METHODS

Sufficient kernels of inbred lines Mo17Ht, Mo20W, N141, and Oh3A to make four replicates of 20 kernels per plate were surface-sterilized for 1 min in 2% NaOCl. Half of the sterilized kernels of each inbred were decapped with a razor blade and the other half were left intact. The kernels were placed on moist filter paper in sterilized petri dishes and sprayed with 4 cc of an aqueous suspension of 20,000 conidia per cubic centimeter of *A. flavus* culture NRRL3357. Kernels were incubated 7 days at 28 C, removed, and half the kernels were washed with a rock-polishing grit of medium grain size by immersing the kernels in a flask of aqueous suspension of grit and gently rotating on a platform shaker until the mycelia were removed from the surface. Rapid, prolonged rotation was avoided to prevent removal of the pericarp. The remaining kernels were not washed in order to leave the external mycelia.

Kernels were dried at 60 C and ground. The extraction was done by a scaled-down version of the AOAC method (1) using $CHCl_3$. Sample extract cleanup on a small silica column and the high-pressure liquid chromatography (HPLC) analyses were done as follows using procedures based on the work of Thean et al (10), Pons and Franz (8,9), and Panalaks and Scott (7): 1) Corn samples were weighed in a hood; 2) 0.5 g of filter aid (Fisher Hyflo Super Cel) was dispensed into a 25-ml screw-cap tube and a 1-g sample of corn was weighed into the tube; 3) 2 ml of water, then 10 ml of $CHCl_3$, were added to the wet sample; 4) a Teflon-lined cap was screwed tightly onto the tube and the sample was shaken for 30 min; 5) the sample was poured through filter paper into a 25-ml Erlenmeyer flask; 6) when the extractant had filtered through, 5 ml of $CHCl_3$ was added to the extraction tube, which was then capped, shaken by hand, and the contents poured over the corn residue in the filter paper; 7) when the extractant and wash had filtered through, the 25-ml Erlenmeyer flask was placed on a small hot plate at low heat to take the extract to dryness under N_2 purge. The dried extract was stored in a freezer, ready for cleanup.

After extraction, the following cleaning-column procedure was employed: 1) Dried extract was dissolved in 500 ml of $CHCl_3$ with the aid of ultrasonic, and 500 ml of hexane was added; 2) the cleanup column was prepared by passing 1–2 ml of hexane through, leaving the level of hexane just at the top of the upper level of Na_2SO_4; 3) the sample was transferred to the column with a Pasteur pipet, then 1 ml of $CHCl_3$:hexane (1:1) was added to rinse the Erlenmeyer flask, and this wash was added to the column; 4) the sample solution was allowed to flow until it reached the level of the Na_2SO_4 layer; 5) the column was washed with 2 ml of hexane followed by 5 ml of anhydrous ethyl ether; 6) aflatoxin was eluted from the cleanup column with 3 ml of $CHCl_3$:CH_3OH (97.3) into a clean, dry culture tube (16 × 75 mm); 7) the sample was taken to dryness in the culture tube at 40 C under a stream of N_2, then stored in a freezer until HPLC analysis was done. The liquid containing the washed-off mycelia was dried and analyzed for aflatoxin.

RESULTS AND DISCUSSION

AFB_1 levels in the wounded-kernel, nonwashed treatment were higher than in the wounded-kernel, washed treatment (Table 1). Mo17Ht and N141 had the highest levels in the nonwashed treatment (935 and 2,193 ppb, respectively). The lowest levels for this treatment were 183 and 581 ppb for Mo20W and Oh3A, respectively.

Mo20W and Oh3A had the lowest levels (117 and 517 ppb, respectively) in the wounded, washed treatment. The highest levels were found in N141 and M17Ht (955 and 611 ppb, respectively). Differences in aflatoxin content among

Table 1. Aflatoxin B_1 (in parts per billion) within kernels of four inbred lines of maize, wounded vs. whole and washed vs. nonwashed[y]

Inbred	Wounded kernels		Whole kernels		$\bar{x}$
	Washed	Nonwashed	Washed	Nonwashed	
Mo17Ht	611	935	174	336	514 b[z]
Mo20W	117	183	120	299	180 c
N141	955	2,193	201	1,260	1,152 a
Oh43A	517	581	167	211	369 bc
$\bar{x}$	550 b	973 a	165 c	526 b	

[y]LSD ($P = 0.01$) for inbred lines within treatments = 117. LSD ($P = 0.01$) = 68 among inbred lines for a common treatment and among treatments for a common inbred line (C.V.% = 77.95).
[z]Numbers within a column or row followed by the same letter are not significantly different according to Duncan's multiple range test for the marginal means.

Table 2. Aflatoxin B_1 (in parts per billion) in wash water from wounded and whole kernels of four maize inbred lines[y]

Inbred	Wounded kernels	Whole kernels	$\bar{x}$
Mo17Ht	12.00	6.50	9.25 a[z]
Mo20W	4.27	5.25	4.76 b
N141	16.00	5.25	10.63 a
Oh43A	2.25	3.00	2.63 b
$\bar{x}$	8.63 a	5.00 b	

[y]LSD ($P = 0.01$) for inbred lines within treatments = 1.11. LSD ($P = 0.01$) = 0.82 among inbred lines for a common treatment and among treatments for a common inbred line (C.V.% = 54.33).
[z]Numbers within a column or row followed by the same letter are not significantly different according to Duncan's multiple range test for the marginal means.

inbred lines were highly significant as were differences between treatments, i.e., wounded vs. nonwounded and washed vs. nonwashed. The inbred lines within treatments interaction was also highly significant, demonstrating that inbreds responded differently to certain treatments. For example, the washing of N141 kernels gave a drastic reduction in aflatoxin contamination, whereas the washing of Oh3A had very little effect on the amount of contamination.

The nonwashed, wounded kernels had significantly more aflatoxin than did the washed kernels, and the washed whole kernels had the least. The washed, wounded kernels and the nonwashed, whole kernels did not differ significantly in aflatoxin content. A greater amount of aflatoxin was recovered from the wash-water fraction from the wounded kernels than from the whole kernels in all instances except for Mo20W.

For the wounded series, the expected external contamination for the washed kernels would be 1,238 ppb (internal contamination 955–2,193 ppb) for inbred N141. The expected external contamination for hybrids Oh3A and Mo20W would be 64 and 66 ppb, respectively. For the whole-kernel series, the expected external contamination would be the highest for N141, 1,059 ppb (internal contamination 201–1,260 ppb). In this instance, the surface mycelia were a major contributor to the AFB_1 content of the kernels. The surface-contamination for Mo17Ht and Mo20W was about the same (162 and 179 ppb, respectively). The lowest expected surface-contamination would be 44 ppb (internal contamination 167–211 ppb) for Oh3A.

The mean aflatoxin level in the wash water from N141 and Mo17Ht (10.63 and 9.25, respectively) was significantly greater than that from Mo20W and Oh3A (4.76 and 2.63, respectively) (Table 2). This followed the same trend as the levels in the inbreds in the nonwashed treatments. The important fact demonstrated here is that the level of aflatoxin in the wash water was not indicative of the actual surface-contamination, as shown in Table 1. Apparently, AFB_1 was lost or destroyed in the evaporation of the wash water and could not be recovered in the analytical process.

In summary, whole kernels produced sufficient levels of AFB_1 to identify differences between genotypes in the search for those with minimal levels. External conidia and mycelia constituted significant amounts of the toxin kernel content. However, whole kernels had lower levels of external and internal toxin than the wounded kernels, indicating that the pericarp and aleurone layers contribute to the defense of the kernel against the fungus. The differences in AFB_1 levels between genotypes portrayed in the wounded series suggested that the chemical composition of the kernels, as yet undefined, must be involved.

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