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# Preharvest Aflatoxin Contamination: Effect of Moisture and Substrate Variation in Developing Cottonseed and Corn Kernels

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**Variations in moisture and substrate in preharvest corn kernels and cottonseed were linked with the ability of *Aspergillus parasiticus* to infect the seed and produce aflatoxin. Osmotic pressures and moisture content (MC) levels of developing starch-rich corn kernels and lipid-rich cottonseed were determined. For in vivo studies, corn kernels and cottonseed were inoculated with *A. parasiticus* conidia and retained on plants through maturation. For in vitro studies, samples of corn kernels and cottonseed were collected at various stages, sterilized, inoculated, incubated for 2 weeks, and assayed for toxin. Aflatoxin levels were highest in corn kernels inoculated at 28 days postflowering (52% MC) in both the in vivo and in vitro tests. Toxin concentrations in cottonseed were greatest with inoculation at 35 days postflowering (70% MC) in seed retained on the plant, but toxin accumulation continued to increase with the maturity of the seed inoculated in cottonseed used in the in vitro trials. Moisture and substrate conditions in the midrange of seed development provided optimum conditions for fungal development and toxin production in seed retained on the plant.**

A number of environmental factors have been linked to the infection of developing cottonseed (*Gossypium hirsutum* L.) and corn kernels (*Zea mays* L.) by *Aspergillus flavus* Link ex Fr. and *Aspergillus parasiticus* Speare (5, 6). The process is confounded by a myriad of variables, but moisture is particularly critical. Aflatoxin-producing fungi were initially considered storage fungi since they exhibited an ability to develop at reduced moisture levels relative to the levels required for field fungi (2, 3). However, subsequent discovery of the extensive occurrence of aflatoxin-producing species in developing crops required reassessment of the field-storage concept (6). The xerotolerance exhibited by the aflatoxin-producing species does not restrict development of the microbes in the field under appropriate conditions. The interaction of moisture availability with other factors appears to modulate the agroecosystem niches of developing cottonseed and corn kernels in a way that, in some instances, favors development of *A. flavus* and *A. parasiticus* and elaboration of aflatoxin in the seed before harvest (4–6).

In addition to moisture, other factors, such as the chemical composition of developing seed, determine the physiological responses of invading fungi (6). Moisture-substrate interactions in developing seed significantly influence susceptibility to fungal infection and elaboration of secondary metabolites (5, 6). Dynamic processes in developing seed convert monomeric substances such as sugars and amino acids into polymers in the natural context of moisture diminution that occurs during maturation (10). In addition, moisture-substrate interactions in developing seed fluctuate widely between diverse crop types such as the oily seed of cotton and the starchy endosperm seed of corn and provide unique environments for microbes (2, 3).

The objective of the current investigation was elucidation of maturity-dependent factors in developing corn kernels and cottonseed that uniquely affect growth of an aflatoxin-producing fungus and elaboration of the toxin before harvest. Material retained on plants during maturation was compared with samples that were removed from plants and used as axenic-culture substrates for *A. parasiticus*. Moisture availability in corn kernels and cottonseed was used as a key determinant in the host plant-fungal interaction.

## MATERIALS AND METHODS

Cotton (Stoneville 213) and corn (Pioneer 3369A) were grown in test plots in New Orleans, La. For in vivo studies, corn ears and cotton bolls were inoculated with *A. parasiticus* NRRL 2999 conidia at various maturity stages. Inoculations were initiated at 21 days postflowering. Corn inoculations comprised one 0.2-ml injection of a conidial suspension ($10^8$ conidia per ml) into the base of the silk bundle of corn ears and two 0.1-ml injections of the suspension through the husk into the area of developing kernels. Cotton bolls were injured at the carpellary suture with a razor blade, and dry *A. parasiticus* spores were dusted onto the wounded area with a small paint brush. Corn ears and cottonseed were retained on plants until maturity, i.e., corn kernels at 16% moisture and cottonseed at 12% moisture.

The potentials of cottonseed and corn kernels as substrates for in vitro production of aflatoxin were determined at various stages of development. Approximately 30 g of seed was collected for each fermentation and placed in a 300-ml Erlenmeyer flask; duplicate flasks were used for each test period. To avoid disruption of outer seed integuments, substrates were sterilized without heating by adding 0.2 ml of propylene oxide to each flask and incubation for 12 h in an airtight container; residual propylene oxide was removed in a vacuum oven at 40°C for 60 min. Sterility was initially verified by placing treated seeds on potato dextrose agar and incubating them at 28°C for 7 days. Flasks were capped with sterile closures and inoculated with 0.2 ml of an *A. parasiticus* conidial suspension ($10^8$ spores per ml). Loss of moisture during removal of residual propylene oxide was monitored gravimetrically, and sterile water was added to achieve initial moisture levels. Flasks were incubated statically for 2 weeks at 28°C.

The moisture content (MC) levels of corn kernels were measured periodically by collecting approximately 50 g of kernels for each sample, determining the wet weights,

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TABLE 1. Developing corn kernels: variations in moisture levels, osmotic pressures of expressed sap, and suitabilities as substrates for aflatoxin production[a]

| Time of inoculation[b] (day post-flowering) | MC (% ± SD) | Osmotic pressure (kPa) | Aflatoxin $B_1$ (ng/g) | |
|---|---|---|---|---|
| | | | In vivo | In vitro |
| 21 | 58 ± 1 | 450 ± 10 | 57 ± 27 | 258 ± 93 |
| 28 | 52 ± 2 | 591 ± 18 | 141 ± 19 | 10,970 ± 1,363 |
| 35 | 41 ± 1 | 683 ± 22 | 59 ± 6 | 10,090 ± 697 |
| 42 | 31 ± 1 | — | 25 ± 20 | 392 ± 138 |
| 49 | 28 ± 0.1 | — | ND | 5 ± 1 |

[a] MC levels of kernels at various maturity stages were determined by the oven-dry method, and osmotic pressures were determined by measurement of freezing-point depression of kernel saps. Three independent determinations were made for each test. —, Inability to express sap because of seed maturity; ND, none detected.

[b] Kernals on test ears for in vivo tests were inoculated with *A. parasiticus* conidia at the designated maturity stages, and ears were retained on plants until maturity (16% moisture). For in vitro studies, kernels were removed from ears at the designated maturity stages and used as substrates for axenic culture of *A. parasiticus* in 14-day incubations at 28°C.

spreading kernels in large ceramic dishes, drying kernels for 24 h in a 104°C oven, and determining the dry weights. About 20 g of cottonseed for each sample was collected from bolls and delinted, and moisture level determinations were made in the same manner as for corn. Water activity determinations were made on exudates expressed from developing seed. Excised seeds were ground with a mortar and pestle and filtered through cheesecloth. The filtrates were examined for freezing-point depression in an Osmette A (Precision Systems, Inc., Bradbury, Mass.) automatic osmometer. Reference solutions with known solute concentrations were used to standardize the instrument. Tests for moisture and freezing-point depression were carried out in three independent replications for each determination.

For aflatoxin assays, mature seeds from the in vivo tests were ground in a hammer mill before methylene chloride extraction. For the in vitro studies, the contents of each fermentation flask were transferred to a Waring blender (Waring Product Co., New Hartford, Conn.) with 150 ml of methylene chloride and ground for 2 min. The macerate was filtered, and the filtrate was evaporated to the desired volume. For the aflatoxin assay, mature seeds were ground in a hammer mill before methylene chloride extraction. Aflatoxin levels were determined by the procedures established by the Association of Official Analytical Chemists (1). Quantities of aflatoxin were determined on activated thin-layer chromatographic plates coated with 0.5 mm of Adsorbosil-1. Plates were developed in ether-methanol-$H_2O$ (96:3:1, vol/vol/vol) in unequilibrated tanks, and fluorescent spots were measured densitometrically (1).

## RESULTS

Developing corn ears and cottonseed bolls were inoculated at various stages of development with *A. parasiticus* conidia to elucidate the association between moisture and substrate constituents of seed and the ability of the fungus to establish infection sites and produce aflatoxin. Comparisons were made of the effect of water availability at various maturity stages on *A. parasiticus* development in seed retained on the plant until maturity versus seed removed from the plant and used in axenic fermentations. Seed removed from the plant was sterilized with propylene oxide to eliminate the heat-mediated disruption of outer seed integuments.

During the period 21 to 49 days after flowering, corn kernel MC decreased from 58 to 28% (Table 1). The osmotic pressure of seed sap increased from 450 kPa at 21 days postflowering to 683 kPa at 35 days postflowering. No sap could be extruded from either corn kernels or cottonseed at 42 or 49 days postflowering. Maximum levels of aflatoxin in the in vivo field-inoculated corn kernels were detected in those inoculated at 28 days postflowering (52% MC); aflatoxin was not detected in kernels inoculated at 49 days postflowering. A similar pattern of peak production was observed in 28-day-old kernels removed from the plant and used in the in vitro tests as a substrate for axenic culture of *A. parasiticus*. Although both 28-day-old (52% MC) and 35-day-old (41% MC) kernels supported elevated aflatoxin $B_1$ levels (>10,000 ng/g), a markedly smaller amount of toxin accumulation (5 ng/g) was detected in the fungal fermentations for samples of kernels inoculated at 49 days postflowering (28% MC).

Maturing cottonseed presented a slightly different pattern of MC and osmotic pressure than corn kernels. During the trial period from 21 to 49 days postflowering, the MC of the seed fell from 83 to 64% (Table 2). In contrast to that for corn kernels, the osmotic pressure for cottonseed decreased slightly during the 21- to 35-day test period. The pattern of aflatoxin accumulation in cottonseed also varied from that observed for corn kernels. Maximum accumulation in field-inoculated seed occurred in seed inoculated at 35 days postflowering, with markedly lower aflatoxin levels detected in seed inoculated at later stages of development. However, the pattern of peak toxin production in cottonseed in the midrange of the maturation period was not observed in the in vitro tests. Cottonseed collected from the plants at various maturity stages and used for laboratory fermentations demonstrated an ability to support elevated levels of toxin production from the mid- to late-maturity stages. The highest level of aflatoxin $B_1$ (19,200 ng/g) in the in vitro fermentations was detected in seed inoculated at 49 days postflowering.

## DISCUSSION

Maximum accumulation of aflatoxin occurred in corn kernels and cottonseed that were inoculated at 52 and 70% MC, respectively, and retained on the plants until maturity. The window of vulnerability is a critical facet of field

TABLE 2. Developing cottonseed: variations in moisture levels, osmotic pressures of expressed sap, and suitabilities as substrates for aflatoxin production[a]

| Time of inoculation[b] (day post-flowering) | MC (% ± SD) | Osmotic pressure (kPa) | Aflatoxin $B_1$ (ng/g) | |
|---|---|---|---|---|
| | | | In vivo | In vitro |
| 21 | 83 ± 0.3 | 437 ± 3 | 1,600 ± 57 | 610 ± 160 |
| 28 | 77 ± 0.4 | 372 ± 8 | 11,070 ± 600 | 7,990 ± 1,980 |
| 35 | 70 ± 1 | 368 ± 14 | 18,704 ± 1,545 | 17,600 ± 3,680 |
| 42 | 69 ± 1 | — | 26 ± 5 | 17,600 ± 7,450 |
| 49 | 64 ± 1 | — | ND | 19,200 ± 5,400 |

[a] MC levels of cottonseed at various maturity stages were determined by the oven-dry method, and osmotic pressures were determined by measurement of freezing-point depression of cottonseed saps. Three independent determinations were made for each test. —, Inability to express sap because of seed maturity; ND, none detected.

[b] Test cotton bolls for in vivo tests were inoculated with *A. parasiticus* conidia at the designated maturity stages, and seeds were retained on plants until maturity (14% moisture). For in vitro studies, cottonseeds were removed from plants at the designated maturity stages and used as substrates for axenic culture of *A. parasiticus* in 14-day incubations at 28°C.

contamination of these crops by aflatoxin. The xerotolerant nature of aflatoxin-producing fungi has been clearly established in previous work with fungus development in stored starchy grain at 17.5 to 20% MC and below 15% MC in oilseeds (2, 3, 6–8, 11, 12). In the current study, the observed development of *A. parasiticus* at moisture levels exceeding 50% introduces provocative questions about the contamination process. Contemporary views of microbial competition in developing plant tissues indicate that field fungi such as *Fusarium*, *Helminthosporium*, and *Alternaria* spp. should predominate in preharvest environments, whereas the more xerotolerant storage fungi such as *Aspergillus* spp. should fill the ecological niches created in the reduced-moisture conditions of storage (2, 3, 6). The observation of maximum *A. parasiticus* production of aflatoxin at field-type moisture levels presents an ecological quandary.

Although moisture determinations are reliable indicators of maturation stages of developing seed, aqueous seed solutions contain a number of solutes that determine actual water activity. Osmotic pressure determinations for developing corn kernels demonstrated an increase in solute concentration during the period between 21 and 35 days postflowering. High levels of aflatoxin were detected in seed inoculated at the two highest levels of osmotic pressure (28 and 35 days postflowering). However, a different pattern of fungal susceptibility was observed in cottonseed. Although MC levels declined during the period from 21 to 35 days postflowering, the seed osmotic pressure decreased. The results demonstrate a marked difference between the starch grain of corn and the lipid-rich cottonseed in the incorporation of solutes into cellular macromolecules during seed maturation (5, 10). Although the osmotic pressures of seed saps did not appear to directly influence *A. parasiticus* development, the maximum production of aflatoxin in the in vivo tests occurred in corn kernels (52% MC) and cottonseed (70% MC) in the midrange of development. The trial period (21 to 49 days postflowering) covered the period from early kernel development to full dent stage in corn and early seed growth to about 1 week before boll opening in cottonseed.

In vitro substrate evaluation of developing corn kernels and cottonseed demonstrated distinct differences in fermentation yields of aflatoxin. Corn at stages of maturity with 41 and 52% MC provided the highest toxin accumulations. Since the results paralleled peak production in inoculated seed that was retained on the plants, a maturity-mediated factor in corn was clearly linked to the toxin production process. However, cottonseed as a substrate for axenic culture did not demonstrate peak toxin yields in the midrange of maturity. Instead, peak toxin production was observed through the mid- to late-maturity test period (35 to 49 days postflowering).

The results of the current study suggest that (i) the availability of carbon and nitrogen substrates in early corn kernel and cottonseed development restricts fungal development (9); (ii) a narrow window of ideal conditions for toxin production occurs about midway through development when available moisture, sugar, and amino acid nutrients are best suited to support toxin production (4–6); (iii) in the midrange of development, corn kernels and cottonseed have not developed physical-chemical inhibitors to fungal infections; (iv) water availability in the 600 kPa range of osmotic pressure provides optimum conditions for *A. parasiticus* development in maturing corn kernels (11, 12); and (v) after being removed from plants at 42 to 49 days postflowering, corn kernels retain barriers to fungal penetration, but cottonseed loses the ability to inhibit fungal development.

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