Aflatoxin Contamination in Corn Differs Among Inoculation Techniques

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Abstract
Aflatoxin research in corn (Zea mays L.) usually requires application of inoculum of Aspergillus flavus to soil or plant ears. The pin-bar vs. side-needle or spray vs. solid material inoculations using A. flavus isolate F3W4 (NRRL 30798) were compared in 2004, 2006, and 2007 using three hybrids in two irrigated experiments each year at Stoneville, MS. Both were planted on a silt loam soil in randomized complete block designs with four replications of treatments. Mature ears inoculated by the pin-bar, side-needle, or spray methods were analyzed for aflatoxin. Ears from controls and solid material inoculum treatments were sampled for analysis at plot harvest. Pin-bar inoculation had more aflatoxin in 2004 (551.9 ng/g) and 2006 (305.8 ng/g) than side-needle inoculation (342.2 ng/g and 151.1 ng/g for 2004 and 2006, respectively), which was greater than controls (76.8 ng/g and 21.6 ng/g for 2004 and 2006, respectively). Solid material inoculation did not differ in aflatoxin from controls. Spraying produced the most aflatoxin (344.1 ng/g) only in 2004. Aflatoxin was low in 2007 when timely rainfall, irrigation, and no temperatures ≥ 35°C resulted in only the pin-bar (20.8 ng/g) and solid material (20.6 ng/g) treatments having > 2.0 ng/g of aflatoxin.

Background on Aflatoxin in Corn
Infection of corn (Zea mays L.) grain by the fungus Aspergillus flavus Link. prior to harvest can result in grain contamination with aflatoxin B1, a known potent carcinogen (8). Aflatoxin contamination in corn occurs worldwide posing a serious health threat to people and livestock, especially in less developed nations. In the United States its incidence is most frequent in the south-central and southeastern states but has occurred in the Midwest, especially during years of drought and heat stress (3,15). Losses due to aflatoxin contamination of corn grown in Arkansas, Louisiana, Mississippi, and Texas in 1998 were estimated to be $85 million (US) (18).

Research directed at controlling aflatoxin contamination in corn has basically involved two approaches; developing genetically resistant germplasm, and devising management practices to reduce environmental stresses that predispose corn grain to infection by A. flavus (5). Both approaches encounter the challenge of trying to duplicate or simulate environmental conditions that favor infection by A. flavus and contamination of grain with aflatoxin. Though A. flavus is distributed worldwide (10), pre-harvest infection of corn grain can be sporadic, necessitating inoculation of ears or application of inoculum to soil to study host resistance or cultural practices that may reduce aflatoxin contamination.

Environmental conditions associated with infection by A. flavus and subsequent aflatoxin contamination are drought, air temperatures ≥ 35°C, and high net evaporation rates (3). Other stress factors such as insufficient levels of N, weed control failures, or insect damage have been linked to increased levels of aflatoxin (5). Natural infection of corn by A. flavus occurs in two ways; one is when conidia are blown or carried by insects onto susceptible silks where they germinate and grow down the silk tube onto the developing grain surface (11) or by growing into breaks in the pericarp of the kernel (9,13).
Inoculation techniques used in aflatoxin research include using a pin-bar constructed of a wooden block with three rows of closely spaced sewing needles laced with conidia to wound the ear through the husk (19); a side-needle injection of a conidial suspension into an ear using a tree marking gun and a 14-gauge hypodermic needle; spraying a conidial suspension with a garden sprayer on the silks at anthesis (1); or spreading a solid inoculum such as autoclaved small grain kernels colonized by *A. flavus* onto the soil surface between or within corn rows (2).

Windham et al. (20) reported inoculating commercial corn hybrids using the side-needle technique resulted in consistently higher aflatoxin contamination levels compared to spraying a conidial suspension on the silks at anthesis or allowing natural infection by *A. flavus*. Information comparing inoculation techniques using a pin-bar vs. side needle or solid material inoculum vs. spraying conidia on the silks is limited, especially with isolate F3W4 (NRRL 30798). In screening germplasm for genetic resistance to aflatoxin contamination, wounding kernels with either the pin-bar or side needle is considered by some as an acceptable way of introducing the fungus. However, such invasive procedures in studies to evaluate the influence cultural practices could overwhelm the benefit of these practices in producing sound kernels that might resist infection by *A. flavus*. The objectives of this study were to evaluate four techniques of inoculating corn with *A. flavus* and to determine their usefulness in aflatoxin research. Data on grain yield, grain moisture at harvest, grain bulk density, and kernel weight were collected to determine if differences in these factors might help explain differences in aflatoxin contamination.

**Two Experiments Used to Evaluate Inoculation Procedures**

This study was conducted using two experiments to reduce the possibility of cross-contamination of pin-bar and side-needle inoculations with conidia from the solid material and/or spray inoculation treatments. Experiment 1 included the pin-bar inoculation technique, side-needle inoculation technique, and a non-inoculated control. Experiment 2 consisted of the spray inoculation technique, the solid material inoculation technique using infested wheat (*Triticum aestivum* L.) seed, and a non-inoculated control.

Both experiments were conducted at the Mississippi State University Delta Branch Experiment Station in Stoneville in 2004, 2005, 2006, and 2007. The studies planted in 2005 were lost to Hurricane Katrina. Soil at the site of both experiments was a Dundee silty clay (fine-silty, mixed, thermic Aeric Ochraqualfs) prepared for planting in late winter each year by forming 30-cm raised beds spaced 76 cm apart. The previous crop each year of the study was soybean (*Glycine max* L. Merr.). The design for each experiment was a randomized complete block replicated four times. Individual experimental units were six rows, 9-m long, and consisted of one hybrid and one inoculation treatment assigned at random within each block.

The *A. flavus* isolate F3W4 (NRRL 30798), known to be highly toxigenic was used for all inoculated treatments. Inoculum for the pin-bar, side-needle, and spray treatments was prepared using a technique described by Abbas et al. (1). Briefly, this involved producing conidia on corn cob grits mixed with water and a section of *A. flavus* grown on potato dextrose agar. The inoculum was incubated for 14 to 21 days at 28°C, then conidia were washed from the grits, 20 drops of Tween 20/liter added, strained, and adjusted with sterile-distilled water to 9 × 10^7 conidia/ml. The solid material was prepared using the method described by Abbas et al. (2). It consisted of soaking 1 kg of wheat seed for 12 h in 200 ml of water, autoclaving the mixture at 121°C for 55 min, inoculating it with *A. flavus*, and incubating the mixture at 28 to 30°C until it was determined by laboratory assay that the wheat kernels contained >log 8.0 cfu *A. flavus* per gram of the fungus.

Both experiments consisted of the three corn hybrids AgriGold brand A6333 Bt (AgriGold Hybrids, St. Francisville, IL), Pioneer brand 34B23 (Pioneer Hi-Bred Intl., Johnston, IA), and Hoegemeyer brand 2633 (Hoegemeyer, Hooper, NE). All three hybrids were similar in maturity and two of them (A6333 Bt and 34B23) had good yields with comparable levels of aflatoxin contamination in
previous research (7). Experiment 2 was planted downstream of the irrigation water from Experiment 1 and each experiment was separated by 9 m of planted corn to buffer against possible cross-contamination.

The experiments were planted 19 April 2004, 5 April 2006, and 23 March 2007 at a seeding rate of 100,400 kernels/ha with an expected final population of 85,400 plants/ha. Weed control was achieved by an application of atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine] at 3.6 kg ai/ha and Permit [1,3-dichloro-5-(4,6-dimethoxypyrimidin-2-ylcarbamoylsulfamoyl)-1-methylpyrazole-4-carboxylic acid], Monsanto, St Louis, MO] at the rate of 0.11 g ai/ha. The experiments were cultivated at growth stage V6 as defined by Ritchie et al. (14). Supplemental fertilizer applications of N, P, and K were made according to recommendations for a 10-Mg/ha grain yield per soil analyses conducted at the beginning of the experiments (Pettiet Agricultural Services, Inc, Leland, MS). Both experiments were furrow irrigated beginning at growth stage R1 and continuing until growth stage R6 using a schedule defined by Bruns et al. (6). To reduce natural infection by *A. flavus* which would complicate our comparison of inoculation techniques, irrigation was applied to reduce drought stress in each year.

The pin-bar and side-needle treatments of Experiment 1, and spray inoculation treatment of Experiment 2 were applied to five ears selected at random in both rows two and five of a plot when plants were at mid-silk or growth stage R1 (1,2). The pin-bar was dipped into a pail containing a conidial suspension of *A. flavus*, pressed against the side of the ear, penetrating it, and wounding the kernels. The side-needle method used 3.4 ml of the conidial suspension/ear, and the spray treatment was applied to each ear using 10 ml of the conidial suspension. Plots inoculated with solid material contained conidia laden, wheat seed spread within rows 3 and 4 at growth stage V6 at a rate of 20 kg/ha.

Soon after the plants reached growth stage R6 and just prior to harvest all inoculated plants in the pin-bar, side-needle, and spray inoculation techniques were collected and dried for at least 24 hr at 70°C. Grain was then shelled and ground for aflatoxin analysis using procedures outlined by Sobolve and Dorner (16) and modified as described in Abbas et al. (4). Rows 3 and 4 of all plots in both experiments were machine harvested and grain weights recorded. A 500-g sample was collected for determining grain moisture, bulk density, kernel weight, and aflatoxin levels in solid material inoculated plots and the non-inoculated control plots.

Data from each experiment were analyzed separately using the PROC MIXED procedure of SAS 9.2 (SAS Institute Inc., Cary, NC). Because of the lack of homogeneity in all data, years were treated as a fixed effect in each experiment. The means separations used for these experiments were the least significant difference ($P \leq 0.05$).

**Weather Observations**

Weather data recorded 100 m from the experimental sites showed more total rainfall occurred in 2004 than in 2006 or 2007, but the bulk of it came between 63 DAP (days after planting) and 74 DAP during growth stage R2 (Table 1). Due to the frequency and probable intensity of rainfall in 2004, much of it would have been lost in runoff and unavailable for plant growth later in the season. Heavy cloud cover associated with this period of rainfall greatly reduced sunlight reaching the earth’s surface (12). In 2006 the total amount of water received through rainfall and irrigation was less than 2004 or 2007. In 2006 plants were drought stressed during much of growth stages R1 through R6. Total rainfall and irrigation in 2007 was less than 2004 but more evenly dispersed. Considerable rainfall occurred in 2007 in four separate events beginning 88 DAP and likely alleviated most drought stress that would have occurred during kernel development. In July 2004, 6 days had maximum daily temperatures $\geq 35^\circ$C during growth stages R2 to R6 (12). In June and July 2006, a total of 16 days had maximum daily temperatures $\geq 35^\circ$C, three of which were $\geq 37.5^\circ$C. This encompassed the entire reproductive period from...
growth stages VT to R6. No days with maximum daily temperatures ≥ 35°C were recorded in 2007 until 2 August, well after growth stage R6.

Table 1. Rainfall and irrigation events in two experiments with corn inoculated with *Aspergillus flavus* at Stoneville, MS, in 2004, 2006, and 2007.

<table>
<thead>
<tr>
<th>DAP&lt;sup&gt;y&lt;/sup&gt;</th>
<th>2004 mm</th>
<th>DAP&lt;sup&gt;y&lt;/sup&gt;</th>
<th>2006 mm</th>
<th>DAP&lt;sup&gt;y&lt;/sup&gt;</th>
<th>2007 mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>21-29</td>
<td>124.7</td>
<td>3</td>
<td>65.3</td>
<td>22</td>
<td>47.0</td>
</tr>
<tr>
<td>40-45</td>
<td>52.3</td>
<td>17</td>
<td>49.3</td>
<td>40-44</td>
<td>48.8</td>
</tr>
<tr>
<td>58&lt;sup&gt;z&lt;/sup&gt;</td>
<td>25.4</td>
<td>25</td>
<td>62.7</td>
<td>63&lt;sup&gt;z&lt;/sup&gt;</td>
<td>25.4</td>
</tr>
<tr>
<td>63-74</td>
<td>327.2</td>
<td>36</td>
<td>38.3</td>
<td>73&lt;sup&gt;z&lt;/sup&gt;</td>
<td>25.4</td>
</tr>
<tr>
<td>89-90</td>
<td>34.5</td>
<td>55-58</td>
<td>27.7</td>
<td>82&lt;sup&gt;z&lt;/sup&gt;</td>
<td>25.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>63&lt;sup&gt;z&lt;/sup&gt;</td>
<td>25.4</td>
<td>88</td>
<td>53.1</td>
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<td>25.4</td>
<td>96-101</td>
<td>70.9</td>
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<td></td>
<td></td>
<td>80-82</td>
<td>18.3</td>
<td>107-112</td>
<td>107.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>91-92</td>
<td>29.2</td>
<td>114-116</td>
<td>32.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>112&lt;sup&gt;z&lt;/sup&gt;</td>
<td>25.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>564.1</strong></td>
<td><strong>Total</strong></td>
<td><strong>367.0</strong></td>
<td><strong>Total</strong></td>
<td><strong>436.0</strong></td>
</tr>
</tbody>
</table>

<sup>x</sup> Rainfall and irrigation events apply to both experiments.
<sup>y</sup> Days after planting.
<sup>z</sup> Furrow irrigation events equivalent to 25.4 mm of rainfall.

Yield, and Yield Component Observations

The hybrid × year interaction for grain yield (*data not shown*) was statistically significant (*P* ≤ 0.05) but only because of yield differences among hybrids in 2004, 2006, and 2007. Main effect means for yield in 2007 were 13.8 Mg/ha for both experiments; in 2006 11.0 Mg/ha and 10.2 Mg/ha for Experiment 1 and Experiment 2, respectively; and in 2004 7.8 Mg/ha and 8.0 Mg/ha for Experiment 1 and Experiment 2, respectively. Low levels of sunlight in 2004 between 63 DAP and 74 DAP (21 June and 2 July) (12) would have reduced photosynthesis during the late R2 and early R3 growth stages which may have contributed to the lower yields observed that year compared to those of 2006 and 2007. Less rain and irrigation during kernel development in 2006 likely contributed to the lower grain yields compared to 2007. Significant (*P* ≤ 0.05) yield differences were observed among hybrids in Experiment 2 (*data not shown*) but ranged only from 10.0 Mg/ha to 11.2 Mg/ha. No such differences were observed between hybrids in Experiment 1 (10.8 Mg/ha).

Kernel weights in both Experiments had statistically significant (*P* ≤ 0.05) differences among years and hybrids (*data not shown*) but the minimum and maximum weights among both main effects were only 289 mg/kernel and 327 mg/kernel, respectively. As with kernel weights, grain bulk densities in both Experiments were significantly (*P* ≤ 0.05) different among years and hybrids but these differences only ranged from 710.4 kg/m<sup>3</sup> to 772.2 kg/m<sup>3</sup>, and all values were above the minimum 695.0 kg/m<sup>3</sup> required of corn grain to grade US No. 2 yellow, the most common grade traded (17).

Some Inoculation Methods Increased Aflatoxin Levels

Inoculation treatment × year interactions were statistically significant (*P* ≤ 0.05) for aflatoxin levels in both Experiments. Compared to the non-inoculated controls, grain aflatoxin levels for Experiment 1 in 2004 were greater with both pin-bar and side-needle inoculated treatments, and in 2006 with the pin-bar method only (Table 2). Except for the non-inoculated control, aflatoxin contamination in Experiment 1 was greater in 2004 than in the following two...
years. However, in both 2004 and 2006, regardless of the treatment, aflatoxin contamination exceeded the 20 ng/g maximum allowable levels set by the US FDA for food corn or corn entering inter-state commerce (8).

Table 2. Concentrations of aflatoxin in two separate corn experiments inoculated with Aspergillus flavus using the side-needle vs. pin-bar or spray vs. solid material methods of inoculation at Stoneville, MS.\(^x\)

<table>
<thead>
<tr>
<th>Year</th>
<th>Experiment 1(^y)</th>
<th>Experiment 2(^y)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentrations of aflatoxin (ng/g)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>non-inoculated</td>
<td>side-needle</td>
</tr>
<tr>
<td>2004</td>
<td>76.8</td>
<td>342.2</td>
</tr>
<tr>
<td>2006</td>
<td>21.6</td>
<td>151.1</td>
</tr>
<tr>
<td>2007</td>
<td>1.0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

\(^x\) Means of 3 hybrids (A6333Bt, H2633, and 34B23) and four replications. Conidial source F3W4 (NRRL 30798).
\(^y\) For both experiments, means within a column LSD at \(P \leq 0.05 = 124\) and within a row 162.

In Experiment 2 in 2004 the spray inoculation treatment had more aflatoxin than either the non-inoculated control or the solid material inoculation treatment (Table 2). The spray inoculation treatment also produced more aflatoxin contamination that year than in 2006 or 2007. In 2006 the solid material treatment produced more aflatoxin contamination than in 2007 but not in 2004. Also in 2006 and 2007, there were no differences in aflatoxin levels between the inoculation treatments and the non-inoculated control. Aflatoxin contamination levels for 2004 and 2006 in Experiment 2 were similar to those in Experiment 1 by exceeding the maximum allowable aflatoxin level of 20.0 ng/g.

In both Experiments in 2007, except for the pin-bar treatment (20.8 ng/g) and the solid material treatment (20.6 ng/g), aflatoxin contamination was < 2.0 ng/g. The previously reported rain events and lack of temperatures \(\geq 35^\circ\text{C}\) during the 2007 growing season most likely were responsible for the low levels of aflatoxin observed. These data demonstrate the importance of drought and heat stress to pre-harvest aflatoxin contamination in corn.

Based on data from these two experiments, the pin-bar inoculation method was effective in producing aflatoxin contamination levels \(\geq 2.0\) ng/g. The side-needle method may not be quite as effective as evidenced in 2006 and 2007. Both the spray and solid material inoculation methods in Experiment 2 did not appear to be any better in producing aflatoxin contamination. Because of the presence of conidia, the solid material inoculation method presented a greater opportunity than other methods for wind or insects to transport inoculum to plants outside the treated area. Both the spray and solid material inoculation methods appear to offer a means of insuring the presence of A. flavus in experiments where kernel wounding is not desired; however, plot to plot contamination would be a concern. With respect to studying aflatoxin in corn it appears that soil moisture levels sufficient to alleviate drought stress during growth stages R1 through R6, combined with a lack of ambient temperatures \(\geq 35^\circ\text{C}\) will likely inhibit aflatoxin production, regardless of the inoculation method used. At least drought stress would need to be induced if ear infection with A. flavus and aflatoxin contamination is desired.

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