MATERIALS AND METHODS

Conidial inoculum to naturally contact, aminate, maize in storage did not prevent aflatoxin formation (Seitzer et al., 1982). We wanted to assess the effect of A. niger on aflatoxin formation by A. flavus in preharvest maize where the bulk of aflatoxin accumulation occurs (Jones et al., 1981; Shotwell, 1977).

AFLATOXIN FORMATION IN PREHARVEST MAIZE EARS COINOCULATED WITH ASPERGILLUS FLAVUS AND ASPERGILLUS NIGER

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ABSTRACT

Aspergillus flavus and Aspergillus niger commonly co-occur in preharvest maize and one report suggests that this association promotes a greater incidence of aflatoxin. Other studies have shown that Aspergillus niger can prevent, or substantially reduce, aflatoxin formation by Aspergillus flavus in autoclaved maize kernels, and this led us to examine the effect of A. niger on aflatoxin formation by A. flavus in preharvest maize where the bulk of aflatoxin accumulation occurs. Samples of kernels simultaneously wound-inoculated with equivalent numbers of A. flavus and A. niger conidia showed substantial aflatoxin contamination (mean = 9900 ppb) even though significantly more (P = .04) aflatoxin was produced in kernels wound-inoculated only with A. flavus (mean = 36,700). These same wounded kernels had a mean sample pH of 5.45 ± 0.44, well above substrate acidity levels (<pH 3.4) reported to prevent aflatoxin formation in autoclaved maize meal.

Key Words: Aspergillus flavus, Aspergillus niger, aflatoxin formation, maize.

Aspergillus flavus Link; Fr. and Aspergillus niger van Tieghem are widespread colonists of kernels in developing maize ears and infect both wounded and uninjured kernels (Taubenhaus, 1920; Heseltine et al., 1981; Cuero et al., 1985). Both fungal species frequently infect the same kernel (Fennell et al., 1973; Heseltine et al., 1981). Bothast et al. (1976) suggested that A. niger may play a "critical role" in aflatoxin production by A. flavus since the two fungi were commonly isolated from samples of maize kernels where aflatoxin was detected. For example, propagule counts of A. flavus and A. niger closely paralleled the occurrence of aflatoxin in truckloads of maize kernels that had been stored on farms (Heseltine et al., 1975). However, independent investigators have shown that production of aflatoxin by A. flavus was inhibited by A. niger when these fungi were co-inoculated onto autoclaved peanuts (Ashworth et al., 1965), autoclaved rice (Tsubouchi et al., 1980), or autoclaved maize (Wicklow et al., 1980). In the latter example, A. niger did not prevent A. flavus from colonizing the kernels, as evidenced by the fact that each fungus had sporulated in patchy sectors over an equivalent area of the kernel surface. Horn and Wicklow (1983) determined that A. niger can lower the substrate pH of autoclaved maize kernels sufficiently (i.e., <pH 3.7) to suppress aflatoxin production. Applications of A. niger conidial inoculum to naturally A. flavus contaminated maize in storage did not prevent aflatoxin formation (Seizel et al., 1982). We wanted to assess the effect of A. niger on aflatoxin formation by A. flavus in preharvest maize where the bulk of aflatoxin accumulation occurs (Jones et al., 1981; Shotwell, 1977).

MATERIALS AND METHODS

A loose-husked hybrid used in the upper midwestern corn belt (Dekalb XL-12) was grown to maturity in a controlled environment room (photoperiod 14 h; temperature 30 ± 1 C da/20 ± 1 C night; humidity 82 ± 3%) in the Biofarms, University of Wisconsin, Madison (Caldwell et al., 1984). This temperature regime equals 15.8 C thermal units/day, enabling A. flavus to produce high levels of aflatoxin in preharvest maize (Thompson et al., 1980). The controlled environment facility was used to regulate the physical environment of the developing maize ears while eliminating confounding variables such as kernel-contaminating fungi and damage by maize insects. Aspergillus flavus inoculum consisted of a mixture of ten aflatoxin-producing strains, each of which was isolated from maize: NRRL 6536, NRRL 6537, NRRL 6539, NRRL 6540, NRRL 6576, NRRL 6577, NRRL 6578, NRRL 6579,
NRRL 6580. NRRL 6581. *Aspergillus niger* inoculum was represented by a mixture of three strains isolated from maize: NRRL 6408. NRRL 6411. NRRL 13542. Conidial inoculum of individual strains was suspended (1 x 10^8/ml) in sterile 0.01% Triton X-100. and then combined in equal volumes to provide: (1) mixture of ten *A. flavus* strains; (2) mixture of three *A. niger* strains; (3) equal volumes of 1 and 2 (*A. flavus* and *A. niger*).

To inoculate an ear, a sterile toothpick was inserted through the husk, removed, dipped into a spore suspension and reinserted into the wound. Wound inoculations were performed 21 da after silking. Individual ears were inoculated, with two opposing vertical rows of five toothpicks spaced 3.8 cm apart. Six ears were inoculated with the *A. flavus-A. niger* combined inoculum, while controls consisted of six ears wound-inoculated with either *A. flavus* or *A. niger*.

Kernels were removed from dried ears at harvest and segregated as follows: A. wound-inoculated kernels; B. 'first circle', the four kernels immediately adjacent to a wound-inoculated kernel; C. 'second circle', those non-wounded kernels immediately surrounding the 'first circle'; and D. 'all other kernels', representing the remaining kernels on the ear.

Kernels were surface-sterilized in a 2% sodium hypochlorite solution for 1 min, washed twice in sterile water, placed on malt extract agar in Petri dishes (five per plate) and incubated for 6 da at 25 C. The occurrence of *A. flavus* or other fungi growing from the kernels was recorded. Only the uninjured kernels from wound-inoculated ears were plated since *A. flavus* consistently showed visible sporulation on kernels wound-inoculated with this fungus.

The pH of wounded kernels was determined by adding 5 ml of boiled distilled water to each vial containing wounded kernels, and then incubating the vials for 45 min at 28 C on a reciprocal shaker. The kernels were then macerated with a glass rod and the pH was measured with a Beckman pH meter. The electrodes were washed off, and the combined fluid of the sample and electrode washing was freeze-dried before aflatoxin analysis. Portions of each sample of non-wounded kernels were blended 3 min at high speed with distilled water to provide a slurry for pH determination.

All samples were analyzed for aflatoxins by the CB Method approved by the Association of Official Analytical Chemists (1984). Samples of kernels weighing 15-75 g were analyzed by the CB procedure as written, but samples weighing more than 75 g were separated into appropriately sized portions, and results were averaged. Samples weighing 5-15 g were also assayed by the CB Method with the exception that the entire extract was collected and filtered, and the filter paper was washed. The combined extract and washes were concentrated for chromatography on silica gel. Samples weighing less than 1 g were steeped overnight with 25 ml chloroform. 2.5 ml water, and 2.5 g Celite. The mixture was then transferred to a blender by washing with 7.5 ml chloroform. After blending 3 min. the material was filtered and the filter paper washed three times with 100 ml portions of chloroform. The chloroform washes and extract were combined, and concentrated for chromatography on Silica Gel 60 (0.063-0.2 mm) column (0.6 cm i.d. x 20 cm). Quantities of aflatoxins were measured by thin layer chromatography (AOAC. 1984). A series of t-tests were used for comparisons of means. The Analysis of Variance procedure was not used because of the extreme differences in means between cells.

**RESULTS**

*Aspergillus flavus* infected over 94% of the non-wounded kernels from maize ears that were wound-inoculated only with *A. flavus* (TABLE 1). These ears also exhibited substantial aflatoxin contamination in all samples of the wounded kernels (range = 13,200–82,000 ppb). Aflatoxins were also detected in 17 of 18 samples of the uninjured kernels representing the 'first circle' and 'second circle' surrounding the wound, and samples of 'all other' kernels on each ear. This treatment, in contrast to that in which ears were coincoculated with *A. flavus* and *A. niger*, produced more (P = .04) aflatoxins in the wounded kernels. However. differences in aflatoxin levels among treatments were not significant (P > .25) for samples representing the 'first circle', 'second circle', and 'all other' kernels.

When *A. niger* alone was wound-inoculated into the ear, 89-93% of the kernels in samples of the uninjured kernels became infected. No aflatoxins were detected. The mean sample pH of the wounded kernels was 3.90 ± 0.17, with the mean pH values for uninjured kernel samples becoming progressively higher with increasing
**Table I**

Infection (%), aflatoxin contamination and pH of kernels sampled from preharvest maize ears inoculated with *Aspergillus flavus* and *Aspergillus niger*.

<table>
<thead>
<tr>
<th>Treatment and kernel samples</th>
<th>Aspergillus flavus</th>
<th>Aspergillus niger</th>
<th>Both species</th>
<th>Mean sample pH</th>
<th>Aflatoxin analyses</th>
<th>Aflatoxin. ppb*</th>
<th>Mean sample wt (g)</th>
<th>Range</th>
<th>Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wounded kernels</td>
<td></td>
<td></td>
<td></td>
<td>5.82 ± 0.26</td>
<td>0.68</td>
<td>13,200–82,000</td>
<td>36,700</td>
<td></td>
<td></td>
</tr>
<tr>
<td>'First circle'</td>
<td>98</td>
<td>0</td>
<td>0</td>
<td>6.04 ± 0.18</td>
<td>14</td>
<td>154–6290</td>
<td>2100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>'Second circle'</td>
<td>94</td>
<td>0</td>
<td>0</td>
<td>6.13 ± 0.13</td>
<td>26</td>
<td>ND–4720</td>
<td>1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All other kernels</td>
<td>96</td>
<td>0</td>
<td>0</td>
<td>6.12 ± 0.10</td>
<td>96</td>
<td>1–5460</td>
<td>900</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td></td>
<td></td>
<td></td>
<td>3.90 ± 0.17</td>
<td>0.66</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wounded kernels</td>
<td></td>
<td></td>
<td></td>
<td>4.99 ± 0.30</td>
<td>12</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>'First circle'</td>
<td>0</td>
<td>92</td>
<td>0</td>
<td>5.95 ± 0.13</td>
<td>25</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>'Second circle'</td>
<td>0</td>
<td>93</td>
<td>0</td>
<td>6.10 ± 0.11</td>
<td>90</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All other kernels</td>
<td>0</td>
<td>89</td>
<td>0</td>
<td>6.10 ± 0.11</td>
<td>90</td>
<td>ND</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspergillus flavus and A. niger</td>
<td></td>
<td></td>
<td></td>
<td>5.45 ± 0.44</td>
<td>0.60</td>
<td>7960–19,900</td>
<td>9900</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wounded kernels</td>
<td></td>
<td></td>
<td></td>
<td>5.39 ± 0.34</td>
<td>13</td>
<td>260–6150</td>
<td>3500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>'First circle'</td>
<td>74</td>
<td>58</td>
<td>34</td>
<td>6.08 ± 0.18</td>
<td>24</td>
<td>ND–381</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>'Second circle'</td>
<td>67</td>
<td>37</td>
<td>7</td>
<td>6.07 ± 0.06</td>
<td>94</td>
<td>1–557</td>
<td>100</td>
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<td></td>
</tr>
<tr>
<td>All other kernels</td>
<td>44</td>
<td>59</td>
<td>9</td>
<td></td>
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</tbody>
</table>

1 Wound-inoculated at 21 days after silking.
2 Six replicates (ears) per treatment.
3 Mean ± standard deviation.
4 ND = not detected.

Distance from the point of wound-inoculation (Table I). The mean sample pH of the *A. flavus*-infested wounded kernels was 5.82 ± 0.26, a value which fell within the range of pH variation shown for samples of uninjured kernels from the same ears.

Samples of kernels simultaneously wound-inoculated with *A. flavus* and *A. niger* showed substantial aflatoxin contamination (range = 7960–19,900 ppb) and a mean sample pH of 5.45 ± 0.44. The latter value fell within the range of pH variation shown for samples of uninjured 'first circle' kernels from the same ears and for wounded and 'first circle' kernels from *A. flavus* inoculated controls (Table I). *Aspergillus niger* had colonized these ears as evidenced by visible sporulation on wounded kernels and infection of uninjured kernels. Interference with *A. flavus* infection of uninjured kernels by *A. niger* and vice versa is suggested by the reduction in percent kernel infection, when contrasted with control ears inoculated separately with either fungus. Furthermore, considerably fewer individual kernels were infected with both fungal species than would be expected if there was no fungal interference (Table I).

**Discussion**

Our results indicate that *A. niger* did not prevent aflatoxin formation by *A. flavus* when the two fungi were simultaneously wound-inoculated into developing maize ears. We attribute this to the fact that the substrate pH of these wound-inoculated kernels was not lowered sufficiently by *A. niger* to inhibit aflatoxin production. Horn and Wicklow (1983) presented evidence suggesting that inhibition of aflatoxin formation is an effect of *A. niger* lowering substrate pH and not the degradation of aflatoxin. In experiments where *A. niger* and *A. flavus* were simultaneously inoculated onto individual autoclaved maize kernels, there was a 70–96% decrease in aflatoxin production below that of the *A. flavus*-inoculated control. This decrease was lower than expected owing solely to the competitive exclusion of *A. flavus* since conidial sporulation of each fungus occupied sectors of approximately equal area on individual kernels. *Aspergillus niger* lowered the pH of these autoclaved kernels from pH 6.2 (uninoculated control) to pH 3.1–3.7 in the presence of *A. flavus*. In preharvest maize, *A. niger* lowered substrate pH in wound-inoculated kernels when
alone (pH 3.90 ± 0.17), but only slightly in the presence of *A. flavus* (pH 5.45 ± 0.44). Horn and Wicklow (1983) totally suppressed aflatoxin formation simply by adjusting the pH of maize meal disks to pH 3.4 or less. The developing maize ear should represent a buffered system for the organic acid metabolites of *A. niger* (e.g., citric acid; oxalic acid). Therefore, the acidity of wounded and adjacent non-wounded kernels should not be lowered to the levels Horn and Wicklow (1983) recorded for *A. niger*-infested autoclaved kernels.

We did not expect to find evidence for fungal interference in connection with the infection of uninjured kernels by *A. flavus* and *A. niger*. A survey of 238 maize samples, collected at harvest in North Carolina, revealed that *A. niger* colonized 10.9% and *A. flavus* colonized 31.8% of a total 11,900 kernels that were plated (Hesseltine et al., 1981). However, the authors also noted that *A. flavus* and *A. niger* together infected 6.6% of the kernels. A closer examination of their data reveals a positive association between the two molds, because *A. niger* was recorded from 21% and not 10.9% of the 3780 *A. flavus*-infected kernels. Different levels of fungal inoculum or the sequence of ear colonization may explain these patterns of kernel infection in field-grown maize. The nature of the association between *A. flavus* and *A. niger* in agricultural systems should be further investigated to determine if some form of mutualism exists which does not impact directly on aflatoxin-producing ability.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


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