EFFECTS OF MAIZE WEEVILS (Coleoptera: Curculionidae) ON PRODUCTION OF AFLATOXIN B₁ BY ASPERGILLUS FLAVUS IN STORED CORN

JAJUK A. BETI, THOMAS W. PHILLIPS, AND EUGENE B. SMALLEY

ABSTRACT Insects play an important role as facilitators of the aflatoxin-producing fungus, Aspergillus flavus Link, in both preharvest and postharvest corn. The current study investigated the role of maize weevils, Sitophilus zeamais Motschulsky, in enhancing aflatoxin B₁ content in stored corn. In laboratory experiments, aflatoxin B₁ content was quantified with an indirect enzyme-linked immunosorbent assay (ELISA) on corn following artificial infestation with adult weevils that had each been topically treated with 100 spores of A. flavus. Corn kernels infested with A. flavus-contaminated weevils had significantly higher levels of aflatoxin B₁ than A. flavus-inoculated corn without weevils. The presence of maize weevils resulted in increased kernel moisture content during incubation, and grain moisture was positively correlated with aflatoxin content across treatments receiving spores. Aflatoxin B₁ levels were higher in corn treated with fungus-contaminated weevils compared with corn that was mechanically damaged and inoculated with spores, which in turn had more aflatoxin than undamaged corn treated with spores. Aflatoxin B₁ content in corn increased with time of weevil exposure from 7 to 21 d, but decreased after 28 d of exposure. Aflatoxin levels in infested corn increased significantly with increased numbers of A. flavus-contaminated weevils. Maize weevils carried spores both internally and externally; however, substantial numbers of spores were intimately associated with the exoskeleton of adult weevils. These findings indicate that maize weevils facilitate the growth of A. flavus and aflatoxin production in corn by increasing surface area susceptible to fungal infection and increasing moisture content as a result of weevil metabolic activity. Weevil activity can have a profound effect on postharvest aflatoxin production even though little initial inoculum is present.

KEY WORDS Sitophilus zeamais, mycotoxins, stored products, maize, fungi

AFLATOXINS IN STORED commodities represent serious health hazards to humans and animals. Consumption of grain contaminated with aflatoxins may cause acute and chronic toxicosis, liver damage, cancer, or death (Wilson 1978). Aflatoxins are polyfunctional bisfuranocoumarins produced as secondary metabolites by certain isolates or strains of Aspergillus flavus Link ex Fries and A. parasiticus Speare (Diener et al. 1987). Toxin production may vary among strains within each species. High producers are common in the tropics and subtropical regions, whereas nonproducers are most common in temperate regions (Diener and Davis 1969). A. flavus is a common preharvest and postharvest associate of agricultural commodities worldwide, and exists saprophytically on dead plant material. Aflatoxin contamination in corn, Zea mays L., is almost exclusively caused by strains of A. flavus that typically produce both aflatoxin B₁ and B₂ (Cole and Cox 1951). Among the several aflatoxins, aflatoxin B₁ is recognized as the most biologically active (Diener et al. 1987).

Insects have been implicated in facilitating the spread of A. flavus and subsequent production of aflatoxins in corn. Spores of A. flavus have been found inside and on the bodies of corn earworms, Helicoverpa zea (Boddie), European corn borers, Ostrinia nubilalis (Hübner), and maize weevils, Sitophilus zeamais Motschulsky, captured in and around corn fields (McMillian 1983). Although infection of corn ears may occur through the silk, damage to the corn ear and kernel pericarp, either mechanically or biologically, provides direct infection courts for A. flavus and facilitates its proliferation. Although spores may be wind disseminated, spores carried by insects provide directed inoculation at damage sites. Wicklow (1991) described a scenario whereby overwintering nitidulid beetles acquire spores of A. flavus from sclerotia in the ground, then fly to corn plants where they
infect corn ears by passing through feeding holes made by corn earworms or other ear-damaging insects.

Grain weevils in the genus *Sitophilus* can be associated with infection of *A. flavus* in stored corn. The maize weevil, *S. zeamais*, can migrate between field and storage, and is commonly found infesting corn in the field before harvest (Dix and All 1985). Increased contamination of postharvest ear corn with *A. flavus* was directly related to the level and duration of *S. zeamais* infestation (Dix and All 1985; McMillian et al. 1980, 1987). The rice weevil, *S. oryzae* (L.), was shown to facilitate spread of aflatoxin in stored wheat and barley (Sinha and Sinha 1990, 1991).

The objective of the current study was to define more critically the role of maize weevils in enhancing aflatoxin B<sub>1</sub> content in stored corn. We used a model laboratory system to determine the effects of weevil infestation, mechanical damage, infestation time, weevil density, and grain moisture content on aflatoxin levels in stored corn. The potential of maize weevils to serve as vectors of *A. flavus* was also investigated.

**Materials and Methods**

**Insects, Fungi, and Corn.** Maize weevils were collected from corn in storage on a farm in Waukaue, WI, in September 1990. The weevils were reared in glass jars on shelled corn equilibrated to 15% moisture content in a growth chamber maintained at 26°C and 80% RH under a photoperiod of 16:8 (L:D) h. Adult weevils for the experiments were sieved from rearing jars within 1 wk of emergence. The weevils were surface sterilized in a solution of 0.25% sodium hypochlorite for 2 min and rinsed with sterile water. Surface-sterilized weevils were immobilized on a chilled petri dish for topical application of aqueous *A. flavus* spore suspensions.

After surveying several strains of *A. flavus* that varied in their ability to produce aflatoxin, we chose strain NRRL 6432 from the fungus collection at the USDA National Center for Agricultural Utilization Research in Peoria, IL, for all the studies described below. *A. flavus* was grown on potato dextrose agar (PDA) for 5 d at room temperature (22°C) and then stored at 6°C until used. Spores were suspended in sterile water containing 0.05% Tween 20 and adjusted to 100 spores per microliter with a hemacytometer.

Clean hybrid picker-shelled yellow-dent corn used for these experiments was recently harvested from the University of Wisconsin experimental farm in Arlington, WI. The corn was surface sterilized by soaking in a 0.5% sodium hypochlorite solution for 2 min, rinsing with sterile water, and drying in a laminar-flow hood to 15% moisture content as measured by a Steinlite SS250 moisture tester. Experimental units consisted of 20 g of corn in 60-ml sterile glass jars with plastic screw caps that were loosely applied for ventilation. All treatments were applied to experimental jars of corn in randomized complete block designs. The moisture contents (percentage of wet weight) of corn at the end of experiments was determined on 5-g samples from each jar using wet weight–dry weight comparisons following drying in an oven at 100°C for 72 h.

**Quantification of Aflatoxin.** At the end of each experiment, 5 g of corn from each jar was mixed with 95 ml of 70% methanol and ground at high speed in a blender for 2 min. After the mixture had settled, 0.5 ml of clear supernatant was placed into a 1.5-ml microtube and stored at −20°C until analysis. Aflatoxin content was determined with an indirect competitive enzyme-linked immunosorbent assay (ELISA) method developed by Chu et al. (1987) and modified by Gendloff et al. (1992).

**Maize Weevil–Fungus Interaction.** This experiment was conducted to determine if maize weevils contaminated with *A. flavus* spores could increase aflatoxin levels in corn compared with spores with no insects. Ten replicates were deployed for each of the following treatments: corn only as a control, 10 surface-sterilized maize weevils with no *A. flavus* spores, 10 μl of spore suspension distributed evenly throughout the corn (∼1,000 spores) with no weevils, and 10 weevils each inoculated with 1.0 μl of spore suspension (∼100 spores each). All jars were incubated at 26°C and 80% RH for 30 d, after which aflatoxin levels and moisture contents were determined.

**Effects of Weevils Versus Mechanical Damage.** Ten percent of the corn kernels in 1 set of 10 jars received a wound (1 mm wide by 3 mm deep) with a sterile drill. A preliminary study determined that such damage was similar to that in corn infested with 10 maize weevils for 14 d. Ten microliters of the standard spore suspension, or ∼1,000 spores, was mixed thoroughly with the corn in each jar. A 2nd group of 10 jars received 10 weevils that had been inoculated individually with 1 μl of spore suspension, or ∼100 spores per weevil. A 3rd group of 10 jars received 10 μl of spore suspension (1,000 spores) without mechanical damage or weevils, and a 4th group of 10 jars was left untreated as controls. All jars were incubated at 26°C and 80% RH for 30 d, after which aflatoxin levels and moisture contents were determined.

**Effects of Weevil Exposure Time on Aflatoxin.** Corn in each of 40 jars was infested with 10 maize weevils that had previously been inoculated with 1 μl of spore suspension, or ∼100 spores per weevil. Weevils were removed from 10 of these jars after 7 d, from another 10 jars after 14 d, from a 3rd set of 10 jars after 21 d, and from a 4th set of 10 jars after 28 d of incubation. Corn in a 5th set of 10 jars was thoroughly mixed with 10 μl of spore suspension, or ∼1,000 spores, but had no weevils. Ten jars of corn without insect infestation or fungal inoculation were used as controls. All jars in this experiment were incubated at 26°C, 80%
Effects of Weevil Density on Aflatoxin. Five replicates each of the following 6 treatments were deployed: jars with 20, 10, 5, and 2 maize weevils, a group of jars each receiving an equal number of spores of *A. flavus*, and a group of untreated jars as controls. The number of *A. flavus* spores inoculated on each weevil was adjusted for the different weevil density treatments so that a total inoculum of ≈1,000 spores per jar was achieved. All jars were incubated at 26°C, 80% RH for 28 d, after which aflatoxin levels and moisture contents were determined.

Potential of Maize Weevils as Vectors of *A. flavus*. Maize weevils from corn infected with *A. flavus* were examined for their ability to carry spores. One-week-old surface-sterilized adult weevils were allowed to feed on corn at 26°C, 80% RH that had previously been inoculated with *A. flavus* and displayed substantial conidial production. Five replicates of individual weevils examined for spores in 3 different ways were studied. All replicated treatments were repeated at 4, 8, and 12 d after maize weevils were introduced into the contaminated corn. To determine whether spores were carried internally, individual maize weevils were taken randomly from the corn culture, surface sterilized in 0.5% sodium hypochlorite for 2 min, and crushed in 5 ml of sterile water using a glass rod. To determine the number of spores carried externally on the body, 1 maize weevil was rinsed in 5 ml sterile water with agitation to remove spores. The total spore load for a whole weevil was extracted by crushing a weevil from the *A. flavus*-infected colony in 5 ml of sterile water. These 3 different aqueous solutions (external, internal, and whole body) were diluted 1/10, 1/100, 1/1,000, and 1/10,000 with sterile water, and 0.5 ml of each solution was spread on PDA petri plates. The plates for all treatments were incubated at room temperature for 3–5 d, and the number of *A. flavus* colonies on each plate was counted. *A. flavus* colonies were readily distinguishable from those of other common fungi based on color and morphology. Plates yielding the highest number of clearly distinct colonies were used for determination of the number of spores per weevil preparation.

Data Analysis. Differences in aflatoxin production among treatments within experiments were determined with analysis of variance (ANOVA) followed by the Fisher protected least significant difference test (LSD). Moisture contents of corn samples were analyzed with ANOVA when applicable, as were differences in spore loads among treatments in the vector experiment. Correlation analysis was used to study the relationship between aflatoxin content and grain moisture content in certain experiments. All analyses were performed using SAS (SAS Institute 1980).

Results

Maize weevils with spores of *A. flavus* elicited much higher aflatoxin levels in corn compared to corn inoculated with spores alone (Fig. 1). Corn inoculated with spores only had higher aflatoxin levels than corn infested with surface-sterilized weevils. Interestingly, low but significant levels of aflatoxin were detected in corn infested with surface-sterilized weevils, indicating that some *A. flavus* may still have been associated with these insects. The average moisture content of weevil-infested corn with and without spores was higher (20.4 and 21.2%, respectively) than in corn with spores only and no weevils (17.1%) or untreated corn (17.8%). In treatments receiving *A. flavus* spores (both with and without weevils) there was a significant positive relationship between aflatoxin content and corn moisture content (Fig. 2).

Mechanical injury to corn with a sterile drill preceding inoculation with *A. flavus* spores resulted in a higher aflatoxin content than did inoculation of undamaged corn with spores (Fig. 3), but weevil infestation with spores elicited the highest aflatoxin levels. Corn moisture content was highest in this experiment when weevils were present (19.3%), compared with that for mechanical damage with spores (16.3%) and corn inoculated with spores only (15.2%); moisture content of control corn was not recorded. There was a weak positive relationship between aflatoxin levels and corn moisture content in these treatments (Fig. 4). The reason for lower overall aflatoxin levels in this experiment compared to those in the previous experiment is unknown, but may be caused by lower corn moisture contents (range, 15.2–19.3%) compared with those of the previous experiment (17.1–20.4%). Nevertheless, differences among treatments were consistent with our expectations based on the previous experiment.
Both the length of time spore-contaminated weevils were allowed to infest corn and the number of weevils infesting corn had significant effects on aflatoxin levels. Aflatoxin levels were significantly higher than controls when weevils were allowed to infest corn for as little as 7 d (Fig. 5), and this did not differ from the level following exposure for 14 d. The highest aflatoxin levels occurred following infestation with weevils for 21 d, but these levels declined in corn that had been infested for 28 d. This drop of aflatoxin levels from 21 to 28 d occurred during the same time period when the F₁ generation of adult weevils was emerging from the corn. Aflatoxin levels increased in corn as the number of infesting weevils increased, even though the amount of inoculum (total number of spores) was constant across treatments (Fig. 6).

Spores were carried both externally and internally by weevils that had infested A. flavus-infected corn. More spores were carried externally than internally, but the mean number of spores for whole body extracts was higher than the sum of the means for external and internal spores (Table 1). The number of spores carried by maize weevils, whether internally, externally, or determined from...
whole body extracts, increased with the amount of time spent on the infected corn.

Discussion

Our research clearly demonstrates that maize weevils carrying spores of *A. flavus* are very effective at facilitating the production of aflatoxin B₁ in postharvest corn. Previous work in which insects, *A. flavus*, and stored grain were combined in experiments did not control for levels of inoculum. Dix and All (1987) released maize weevils in containers with corn together with agar plugs colonized by *A. flavus*. Similarly, Sinha and Sinha (1991, 1992) examined the aflatoxin levels in samples of grain that either did or did not contain insects. In our experiments, known concentrations of *A. flavus* spores were applied to previously surface-sterilized weevils and corn infested with these weevils routinely yielded high aflatoxin levels. Because infestation with surface-sterilized weevils resulted only in negligible levels of aflatoxin, it appears that simple transfer of spores from the cuticle of weevil bodies to the corn is sufficient for infection on corn.

The spread of aflatoxin in postharvest corn is a function of mechanical injury to the corn and increased moisture content, both of which result from maize weevil infestation. *A. flavus* will not grow at moisture contents <17% (Sauer and Burroughs 1980), and mechanical damage is known to facilitate fungal infection (Tuite et al. 1985). In our studies in which corn was inoculated with *A. flavus* spores only with no weevils or damage, resulting moisture contents were 15–17% and aflatoxin levels were low or undetected. However, when mechanical injury was made with a drill (Fig. 3), moisture content remained relatively low (16.3%) but aflatoxin levels were substantially higher than in undamaged corn treated with spores. Thus, mechanical damage alone can provide a suitable infection court for the growth of *A. flavus* even though overall grain moisture content is not optimal. Maize weevils are ideal for providing both mechanical damage and increased moisture content. Insect infestation usually causes increased heating and moisture content in stored grains due to metabolic activity of the insects (Mills 1983). Whenever weevils were present we found corn moisture contents of 19–20% by the end of a study. Weevil-caused injury is much more extensive than the experimental damage we made with a drill. Adult weevils damage the pericarp and make feeding holes into the kernel, similar to the damage of our drill. However, feeding by maize weevil larvae results in progressive extension of feeding tunnels throughout the kernels that provides increased surface area for growth of *A. flavus*.

Aflatoxin levels in the maize weevil-*A. flavus* system rely less on the amount of beginning inoculum than on the extent of insect infestation in the corn. Total number of spores inoculated was constant among treatments in our weevil density study, but aflatoxin levels increased as a function of weevil density. Thus more weevil activity, resulting in more damage, more larval feeding, and higher moisture contents, facilitated greater fungal growth and aflatoxin production. A similar relationship was evident in the weevil exposure time study. Weevils allowed to remain on corn longer presumably fed more and laid more eggs than weevils infesting for short periods, thus resulting in more larval activity and a higher moisture content. The

### Table 1. Mean number ± SEM of *A. flavus* spores from contaminated maize weevils carried externally as determined from body washes, internally as determined from crushed bodies of surface-sterilized weevils, or total spore load determined from crushed whole bodies

<table>
<thead>
<tr>
<th>Source of spores</th>
<th>Weevil exposure to <em>A. flavus</em>-contaminated corn, d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4</td>
</tr>
<tr>
<td>External</td>
<td>112,000 ± 25,000A</td>
</tr>
<tr>
<td>Internal</td>
<td>18,000 ± 2,000A</td>
</tr>
<tr>
<td>Whole body</td>
<td>312,000 ± 20,000A</td>
</tr>
<tr>
<td></td>
<td>8</td>
</tr>
<tr>
<td>External</td>
<td>137,000 ± 38,000A</td>
</tr>
<tr>
<td>Internal</td>
<td>17,500 ± 6,000AB</td>
</tr>
<tr>
<td>Whole body</td>
<td>458,000 ± 45,000B</td>
</tr>
<tr>
<td></td>
<td>12</td>
</tr>
<tr>
<td>External</td>
<td>220,000 ± 23,000B</td>
</tr>
<tr>
<td>Internal</td>
<td>43,000 ± 12,500B</td>
</tr>
<tr>
<td>Whole body</td>
<td>613,000 ± 37,000C</td>
</tr>
</tbody>
</table>

Mean values in a row followed by different letter are significantly different (*P* < 0.05, ANOVA and LSD, *n* = 5).
highest aflatoxin content in the exposure time experiment was at 21 d, which would have corresponded with a peak in larval activity before pupation and adult emergence. The reduction in aflatoxin content at 28 d exposure requires further study. Sinha and Sinha (1992) also found that an extended time of incubation with insects resulted in reduction of aflatoxin in stored wheat.

Maize weevils are effective vectors of *A. flavus* spores. Our study suggests that the most important mode of spore transport is by external contamination of the weevil body. We found low but measurable numbers of spores were carried internally, suggesting that maize weevils could contaminate stored corn by defection of spores. Viable spores of *A. flavus* have been found in the guts and feces of several insect species (McMillian 1983). Fecal deposition of *A. flavus* spores by maize weevils could be important in fungal growth because females cover new oviposition holes with a presumed fecal plug (Arbogast 1991). Any inoculum in this plug could germinate and grow inside the kernel with the feeding weevil larva. The effect of fecal transmission of *A. flavus* spores by maize weevils has not been studied. Because whole body extracts of maize weevils contained many more spores than the sum of spores from external and internal extracts, we believe that the majority of spore transmission is caused by external carriage by weevils. *A. flavus* spores were probably intimately associated with the cuticle of our experimental weevils, and the simple body wash to measure externally carried spores could not account for all the inoculum. Our infestation experiments with *A. flavus*-contaminated weevils demonstrated that external transmission of spores is very effective in facilitating growth of *A. flavus* and aflatoxin production in stored corn. Additionally, our work shows that very few spores carried by weevils are necessary to cause infection. Surface-sterilized weevils were usually inoculated with just 100 spores, but our study of vector potential indicates that a single maize weevil can carry over 600,000 spores.

Insect infestation in stored commodities has a profound effect on postharvest contamination with aflatoxin B1. Corn in the field is clearly vulnerable to infection by *A. flavus*, particularly when pressure from ear-infesting insects is high and drying of corn is delayed (Wicklow 1991). However, the potential time available for field infestation is short, the combination of contributing factors (ear-damaging insects, spore-carrying insects, high moisture) patchy in space and time, and the overall inoculum relatively dilute compared with postharvest storage systems. Grain in storage may be at risk of insect and fungal invasion for many months to years. Generally, if grain is stored dry (12–13% moisture content) and kept cool (<20°C), insect infestation and related fungal problems are minimal (Hagstrum and Flinn 1992). But ideal conditions are often not achieved, particularly for farm-stored grain. Residual populations of insects usually remain in farm storages for years, even when bias are empty, because they subsist on grain spillage and debris. *A. flavus* spores are ubiquitous in agricultural environments (Diener et al. 1987), and are probably in highest density in feed and grain storages as a result of suitable substrate and moisture conditions. Thus, specialized insect vectors are not needed to bring *A. flavus* spores into storages because they occur there normally, and our work shows that very little inoculum is needed to begin *A. flavus* infection when insects are present. Stored-grain insect infestations are sometimes manifested as hot spots, in which insect and fungal growth results in metabolic heating and increase in moisture (Mills 1983). Such infestation can serve as focal points for the production of aflatoxins by *A. flavus*, and management of stored grain should address factors that cause these infestations.

**Acknowledgments**

We are very grateful to Elie Gendloff for his instruction and assistance with the ELISA. We appreciate reviews of the manuscript by Rodney Caldwell (University of Wisconsin at Madison, WI) and Donald Wicklow (USDA-ARS, Peoria, IL). Funding for this research was provided by a fellowship to J.A.B. from the National Agricultural Research Base Project II of the Indonesian government, and by USDA-ARS through a research support agreement with the University of Wisconsin-Madison.

**References Cited**


their products. American Association of Cereal Chemists, St. Paul, MN.


Received for publication 26 May 1995; accepted 21 August 1995.