Effect of Temperature on the Preharvest Infection of Maize Kernels by *Aspergillus flavus*

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ABSTRACT

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The effect of temperature on colonization of maize silks and the subsequent invasion of kernels by *Aspergillus flavus* was studied in controlled environments. At the postinoculation temperature regimes of 34/30 C (34 C × 9 hr, 30 C × 15 hr) and 26/22 C, as many as 28 and 2.4% of the kernels, respectively, were infected. Infected kernels were present in all areas of the ear, and neither temperature nor time of inoculation affected the location of infected kernels on the ear. At 34/30 C the fungus entered the ear tip in one day and was present in the base by 4 days. Internal infection of the ear did not occur until 8 days after inoculation, and the percentage of infected kernels increased greatly between 28–32 days, when kernel moisture was <32%. These results demonstrate that the parasitic ability of *A. flavus* is enhanced at high temperature and that, although surface colonization of the kernels occurs early, extensive internal infection does not occur until kernel maturity.

Aspergillus flavus ex Fries Link has been observed before harvest on maize (*Zea mays* L.) in the U.S.A. for many years (3,4,8,26). The fungus was considered an ear pathogen of minor importance and was of little concern until it was shown to produce the toxic compound aflatoxin (24). *A. flavus* is common on maize in the southeastern United States (13,17,28) and in certain years can cause problems in the major maize growing areas of the U.S.A. (16). Although the fungus is considered a saprophyte, it can colonize silks and invade developing kernels (11,14,20,21,22). Jones et al (14) showed that infection by *A. flavus* could proceed in the absence of insects and that more kernels were infected at a high temperature (32–36 C) than at a low temperature (21–26 C). Marsh and Payne (21) showed that the condition of the silk greatly influenced the infection process. The objectives of our study were: to examine critically the effect of temperature and time of inoculation on the infection of kernels by *A. flavus*; to determine the effect of temperature on the rate of colonization of silks and kernel surfaces; and to determine when kernels become infected internally with *A. flavus*.

MATERIALS AND METHODS

This study was conducted in controlled environments at the Southeastern Plant Environment Laboratories, Raleigh, NC (7). Plants of the maize cultivar Gaspe × W103 were grown singly in 20.3-cm plastic pots (400 ml) containing one-third gravel and two-thirds Peat-lite. W103 is an early yellow dent inbred and

Gaspe is a partly inbred line from the accession PI 21279 Gaspe flint. Before inoculation, plants were grown at the temperature regime of 26/22 C (26 C × 9 hr, 22 C × 15 hr) with an interrupted night to provide a long day length. Plants were irrigated twice each day with a modified half-strength Hoagland's solution (7).

Isolates NRRL 3357 and 5T of *A. flavus* were grown on potato dextrose agar at 28 C for 10 days, and conidia were dislodged by flooding the cultures with 0.05% Triton X-100 (Amersham Corp., Arlington Heights, IL). The concentration of conidia was determined by a hemacytometer and adjusted by dilution with 0.05% Triton X-100.

Temperature and time of inoculation study. Time of inoculation treatments were imposed by planting at four dates and inoculating on the same day to provide inoculation times of 1, 2, 3, and 5 wk after silking. Four postinoculation temperature regimes were also imposed to correspond to 13.5 (26/22 C), 16.5 (34/22 C), 18.5 (26/30 C), and 21.5 (34/30 C) thermal units (TU) per day. TU were calculated as ([(day temp. × 9 hr) + (night temp. × 15 hr)]/24) − 10 C. The experiment was a factorial design with each of two replicates grown at a different time. A treatment unit consisted of six plants in the first replicate and 10 plants in the second replicate.

Plants were inoculated by spraying the silks with 0.5 ml of a spore suspension containing 1×10^6 conidia per milliliter of *A. flavus* NRRL 3357. Inoculum was applied with an atomizer, and immediately after inoculation the ears were covered with a plastic bag and then a paper bag. After 3 days the plastic bag was removed, but the paper bag remained until harvest.

Half of the plants were moved morning and evening between two rooms to achieve the four temperature regimes. One room was set to day/night temperatures of 26/22 C and the other to 34/30 C with day/night times of 9/15 hr and an uninterrupted night to give a short day-length effect.

To allow for compensatory growth of the fungus at the lower temperatures, ears were harvested from each treatment after the plants had been exposed to 481 TU. Ears were harvested in the husk and stored at −20 C until analyzed. Kernels were removed from the tip, middle, and base regions of the ear, surface disinfested for 3 min, and plated on malt agar with 6% NaCl (MSA). In the first replicate, the kernels were surface sterilized in disinfestant A, a solution of sodium hypochlorite:water (10:90). The sodium hypochlorite solution used contained 5.25% NaOCl. Kernels from the second replicate were divided into equal samples, and one-half was treated as above and the other half was treated with disinfestant B, a solution of ethanol:sodium hypochlorite:water (10:40:50). The kernels were incubated for 4 days at 34 C and evaluated for sporulation of *A. flavus*. Surface disinfestant B was included in the second replicate to ensure a more thorough surface disinfestation of the kernels. Replicates surface disinfested with solution A were analyzed together to determine treatment differences.

Rate of colonization study. Plants were grown and inoculated as described above except that the ears were inoculated with a tan mutant (5T) of *A. flavus*. Care was taken to prevent the spore suspension from running down the silk channel. After inoculation, half of the plants were placed at the day/night temperature regime of 34/30 C, and the other half were placed at 26/22 C. Three ears were harvested at 1, 2, 4, and 8 days after inoculation from the high temperature chamber and at 4 and 8 days after inoculation from the low temperature chamber.

Harvested ears were taken to the lab, and the external silks and outer husks were removed. A rubber band was placed around the ear tip, and the tip was covered with Parafilm (American Can Company, Greenwich, CT) to prevent the disinfestant from entering the ear. The ears with internal husks intact were surface disinfested by rotating the ear for 3 min in a solution of ethanol:sodium hypochlorite:water (10:20:70).

Each ear was cut into three equal segments (tip, middle, base), and the silks were removed from each segment and plated on MSA. In addition, a cross section of the cob with the developing kernels was removed from the distal end of each section and plated on MSA. Ear tissue was incubated at 34 C for 4 days and evaluated for visible sporulation of the tan color mutant. To test our surface-disinfestation procedure, ears were inoculated as described above, harvested after 2 hr, and surface disinfested. No *A. flavus* was found on any tissue within the husks of the control ears. The rate of colonization study was done twice.

Time of infection study. The study was divided into three experiments. The experimental design was a randomized complete block. Plants were grown as described above, silk inoculated with *A. flavus* isolate 5T, and incubated at the day/night temperature regime of 34/30 C. In the first experiment, 10 ears were harvested at 2, 4, 8, 12, 16, 20, 24, 28, and 32 days after inoculation. Ears harvested from 2 to 8 days were brought to the lab, and the outer husks and external silks were removed. The ears with remaining husks were then surface disinfested by washing the ear with a cotton swab soaked in a solution of ethanol:sodium hypochlorite:water (10:20:70), and the ear was divided into three segments as described for the rate of colonization study. The husks were removed, and silks and cob tissue containing developing kernels and cupules were removed and plated on MSA. The remaining four ears were dried at 60 C for calculation of moisture content. For harvests at 8–20 days, ears were treated the same, but additional sampling was done. All of the kernels were removed from the ears after the initial plating described above, and half of the kernels were surface disinfested for 3 min in ethanol:sodium hypochlorite:water (10:20:70) and plated on MSA. For harvests at 24–32 days, all kernels were shelled from six intact ears, and 100 kernels were surface disinfested and plated on MSA. Surface colonization was not examined in the other two experiments. Kernel moisture was determined by drying 4-g subsamples of kernels from each ear in a forced-air oven at 60 C until the kernels no longer lost weight. Moisture was expressed as a mean of all ears in the treatment. In experiment 2, 10 ears each were harvested every 4 days from day 8 until day 40. In experiment 3, 10 ears each were harvested every 2 days from day 20 until day 36. In both experiments, kernels were removed from the ear, and 100 kernels selected randomly were surface disinfested in ethanol:sodium hypochlorite:water (10:20:70) and plated on MSA. Plates were incubated for 4 days at 34 C and evaluated for sporulation of isolate 5T.

TABLE 1. Influence of four temperature regimes on kernel infeciton of maize ears by *Aspergillus flavus*

Temp. regime[c] (C)	Thermal units	Surface disinfestant A[a] Infected kernels for each ear region (%) Tip	Middle	Base	Whole[d] ear	Surface disinfestant B[b] Infected kernels for each ear region (%) Tip	Middle	Base	Whole ear
26/22	13.5	1.4	3.8	2.0	2.4	2.0	1.3	2.3	2.0
34/22	16.5	7.4	9.7	4.9	6.8	3.3	2.7	2.8	2.8
26/30	18.5	13.7	13.2	11.3	12.7	5.2	3.8	2.8	4.2
34/30	21.5	34.0	27.7	23.0	28.4	16.7	10.3	8.8	12.0
LSD ($P \leq 0.05$)		7.7	8.5	6.7	6.8	7.6	5.0	4.5	4.8

[a] Surface disinfestant A = sodium hypochlorite:water (10:90).
[b] Surface disinfestant B = ethanol:sodium hypochlorite:water (10:40:50).
[c] Temperature regime corresponds to day/night temperatures, with 9 hr of day temperature.
[d] Percentage of infected kernels on whole ear.

TABLE 2. Influence of four times of inoculation with *Aspergillus flavus* on the infection of maize ears

Inoculation time[c]	Surface disinfestant A[a]				Surface disinfestant B[b]			
	Infected kernels for each ear region (%)			Whole[d] ear	Infected kernels for each ear region (%)			Whole ear
	Tip	Middle	Base		Tip	Middle	Base	
1	15.2[e]	16.0	8.7	13.3	10.4	6.4	4.2	7.2
2	15.4	13.0	12.1	13.5	5.2	4.2	5.2	4.9
3	17.4	17.9	11.9	15.7	8.6	3.5	4.1	5.7
5	9.2	7.8	8.9	8.5	2.9	3.8	3.2	3.2

[a]Surface disinfestant A = sodium hypochlorite:water (10:90).
[b]Surface disinfestant B = ethanol:sodium hypochlorite:water (10:40:40).
[c]Inoculation time is weeks after silking.
[d]Percentage of infected kernels on whole ear.
[e]There are no significant differences between treatment means ($LSD_{0.05}$) for values in each column.

RESULTS

Effect of temperature and time of inoculation on infection. Postinoculation temperature greatly affected the percentage of kernels infected with *A. flavus* (Table 1). Few kernels were infected at the temperature of 13.5 per day, but as many as 28.4% of the total kernels were infected at the postinoculation temperature of 21.5 TU. Overall, the percentage of infected kernels detected was less when the kernels were treated in disinfestant B, but the effect of temperature on infection was evident regardless of the surface-disinfestant procedure (Table 1). There was a significant increase in the percentage of infected kernels as postinoculation temperature was increased from 18.5 to 21.5 TU (Table 1, Fig. 1).

The inoculation date did not affect ($P \leq 0.05$) the percentage of infected kernels when the temperature treatments were combined (Table 2). The general trend was for fewer infected kernels in those plants inoculated 5 wk after silking (Table 2). A significant temperature × inoculation date interaction ($P \leq 0.05$) existed only for infected kernels at the ear tip and for the percentage of infected kernels on the whole ear for disinfestant A.

Neither postinoculation temperature nor time of inoculation significantly affected the distribution of infected kernels on the ear (data not shown); however, there was a trend for more infected kernels at the tip of the ear (Tables 1 and 2).

Rate of colonization. *A. flavus* rapidly colonized the silks within maize ears (Table 3). At the highest postinoculation temperature (21.5 TU), the fungus was present in the silks and ear tissue of the ear tip in 1 day, the middle of the ear in 2 days, and in the base in 4 days. Colonization was much slower at the lower temperature (13.5 TU). *A. flavus* was present in the tip region in 4 days and in the base region in 8 days. The fungus was most prevalent on silk tissue at the first harvest date, but in succeeding harvests it became more prevalent on the cupule tissue and the glumes. Rarely was *A. flavus* isolated from the pith tissue.

Time of infection. The rate of surface colonization by *A. flavus* in this study was similar to that found in the rate of colonization study except the fungus colonized a few ears at the base in 2 days. A few kernels were colonized internally by the fungus as early as 8 days after inoculation, but the percentage of infected kernels was low (Table 4). There was an increase in the percentage of infected kernels 28–32 days after inoculation (Table 4.) The exact time of this increase was more dependent on kernel moisture than on harvest date and appeared when kernel moisture fell below 32%. The increase in the number of infected kernels was also associated with kernel maturity. Maximum dry weight of kernels occurred at 28 days in experiment 1 and at 32 days for experiments 2 and 3.

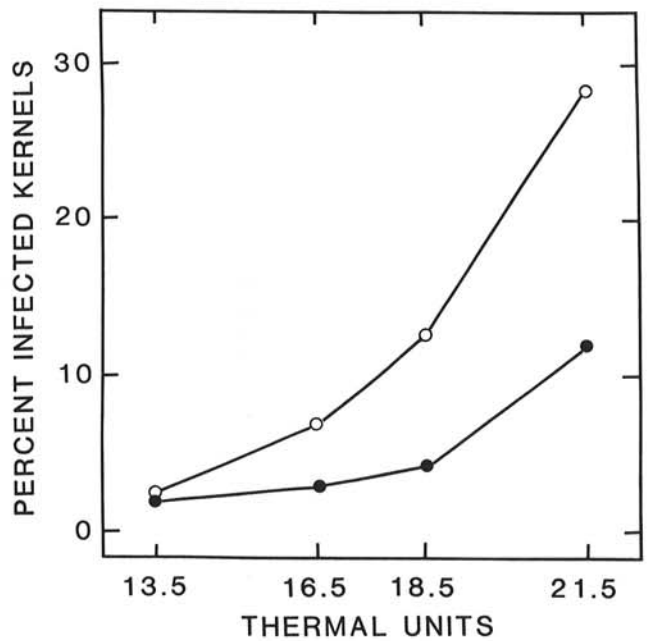

Fig. 1. Percent kernel infection by *Aspergillus flavus* as influenced by postinoculation temperature. Day/night temperatures (9 hr day/15 hr night) and their corresponding thermal units (TU) were: 26/22 C (13.5 TU), 34/22 C (16.5 TU), 26/30 C (18.5 TU), 34/30 C (21.5 TU). Kernels were plated on malt agar with 6% NaCl after surface sterilization with either a 10:90 solution of sodium hypochlorite:water (o—o) or 10:40:50 ethanol:sodium hypochlorite:water (●–●). $LSD_{0.05}$ among treatment means = 6.8% (o—o) and 4.8% (●–●).

DISCUSSION

A. flavus readily infected developing maize kernels under controlled environments. The fungus was able to infect kernels at all temperature regimes examined, but maximum infection occurred at the day/night regime of 34/30 C. These findings agree with those of Jones et al (14), who in greenhouse studies found greater levels of infection at 32–38 C than at 21–26 C, and show that high temperatures are required for an extensive number of kernels to become infected. Increased infection at the higher temperatures in our study is probably related to the high temperature (36–38 C) required for optimum growth of the fungus (19). Our results are also consistent with field observations. High temperature during grain development has been associated with high levels of aflatoxin in the field (2,28), and Fortnum (10) proposed that high temperatures may be the most important environmental factor influencing preharvest infection of maize by *A. flavus* in the southeastern United States.

Time of inoculation did not significantly affect percentage of infected kernels, but the fewest infected kernels occurred on ears inoculated 5 wk after silking. Jones et al (14) reported significantly less infection of kernels when silks of field-grown maize were inoculated 4 wk after silking than when inoculated 1 wk after silking. Our failure to show a significant effect of time of inoculation may be due to differences in the condition of silks between field- and controlled environment-grown maize. In general, silks dry more rapidly in the field than in the phytotron. Marsh and Payne (21) found that silk appearance is a more reliable indicator of susceptibility to *A. flavus* than chronological age.

Jones et al (14) reported that in a greenhouse study, maize ears maintained at a cool temperature had most of the infected kernels at the tip, whereas ears maintained at a warm temperature had most of the infected kernels at the base of the ear. In our study neither temperature nor time of inoculation influenced the distribution of infected kernels on the ear, which indicates that kernels at any position on the ear may be invaded by *A. flavus*.

Sauer and Burroughs (25) have shown that surface disinfestation with sodium hypochlorite alone may not remove all surface contamination due to poor wetting of the kernel surface. We were concerned about effective removal of surface contamination and decided to treat half of the kernels in one replication with a more rigorous surface disinfestant (disinfestant B). Disinfestant B, which contained 10% ethanol as a wetting agent and a higher concentration of sodium hypochlorite, did result in fewer kernels evaluated as positive for infection by *A. flavus*. Kernels treated with disinfestant B, however, showed the same effect of temperature and time of inoculation on infection by *A. flavus* as did those treated with solution A. We are not sure which procedure gave the most accurate evaluation of kernel infection, but both showed the effect of temperature and time of inoculation on infection by *A. flavus*.

Mycelium of *A. flavus* rapidly colonized silks and the kernel surfaces. Internal invasion of a few kernels by *A. flavus* occurred by 8 days after inoculation, but the number of kernels with internal infection increased greatly as kernel moisture fell below 32%. At this moisture, kernels had reached maximum dry-weight accumulation and therefore were physiologically mature. Most hybrid lines of maize reach physiological maturity at 30–32% kernel moisture (1). Thus, physiological and structural changes associated with kernel maturity may be related to the increased susceptibility to invasion by *A. flavus*.

TABLE 3. Frequency of colonization of inoculated maize silks and ears by *Aspergillus flavus* isolate 5T at two temperatures

Temperature[b] (C)	Days after inoculation	Number of ears out of six colonized[a]					
		Tip		Middle		Base	
		Silks	Ears[c]	Silks	Ears	Silks[d]	Ears
34/30	1	4	1	0	0	0	0
	2	2	4	2	3	0	0
	4	2	4	1	2	0	2
	8	4	5	2	4	0	2
26/20	4	5	3	0	0	0	0
	8	4	2	2	2	1	2

[a] Data presented represent two replicates in time.
[b] Temperatures are day/night temperatures, with 9 hr day and 15 hr night.
[c] The designation *ear* refers to cob tissue with developing kernels and cupule tissue.
[d] Very few silks were present in the base of the ear.

TABLE 4. Moisture content and percentage of maize kernels infected with *Aspergillus flavus* isolate 5T at 14 harvest days after inoculation

Days after inoculation	Exp. 1		Exp. 2		Exp. 3	
	% I[a]	MC[b]	% I	MC	% I	MC
8	2.2	67.5	0.2	58.4	...	...
12	3.9	51.8	0.1	45.4	...	...
16	0.5	44.4	0.1	37.6	...	...
20	0.3	39.8	0.6	35.6	0.6	39.4
22	...[c]	...	...	...	0.5	38.8
24	2.0	32.4	0.4	33.7	0.9	38.2
26	...	...	...	...	0.1	36.4
28	25.6	25.9	1.9	31.0	4.3	32.6
30	...	...	...	...	5.5	31.9
32	56.0	16.4	16.2	21.6	17.9	30.1
34	...	...	...	...	26.7	26.8
36	...	...	12.3	14.3	22.2	23.0
38	...	...	...	...	...	...
40	...	...	7.6	10.2	...	...
LSD ($P \leq 0.05$)	11.9		8.8		14.3	

[a] Percentage of kernels with visible *A. flavus* after surface disinfestation and 4 days incubation at 34 C on malt agar with 6% NaCl.
[b] Percent moisture content of kernels at harvest.
[c] Sample not taken.

Other studies demonstrated that ear-inhabiting fungi infect late in the maturity of the kernel. Koehler (15), for example, found extensive colonization of kernel surfaces by *Fusarium moniliforme* Sheld. early in the season, but little internal infection until kernel moisture was lower than 34%. Johann (12) also found that infection of maize kernels by *Diplodia maydis* (Berk) Sacc. occurred late in the development of the kernel. She observed the entrance of *D. maydis* into the pedicel region and penetration through the hilar region. Although the closing layer (i.e., hilar layer, black layer) of the hilum was impermeable to the fungus, she found cases in which the fungus entered the kernel before the closing layer was formed or in cases of delayed or incomplete juncture of the closing layer and the suberized membrane of the testa. In fact, she observed that lines susceptible to the fungus had a delayed or less effective closing of the hilar orifice.

Similarly, Salama and Mishricky (23) suggested that *F. moniliforme* enters the same region in immature kernels. The pathway of colonization by *A. flavus* into maize kernels is not known, but ample evidence (9,20,21) suggests that it also enters through the pedicel region. One could speculate that biochemical resistance may be low at the time of maximum susceptibility due to reduced metabolism, and morphological resistance may not be fully expressed due to incomplete formation of the hilar layer. If this hypothesis is true, moisture content may be more of an indicator of maturity than a direct factor influencing infection.

Optimum temperature for infection by *A. flavus* appears to be higher than that for aflatoxin production. Aflatoxin was rapidly formed in wound-inoculated kernels at the day/night temperature of 30/26 C, but temperatures above this did not significantly increase toxin production (27). Similarly, in vitro production of aflatoxin has been shown to be maximum at 25 C (6,8). There is also an indication that the optimum moisture for toxin production may be higher than that for infection. Lillehoj (18) found that maximum aflatoxin production occurred in field-grown maize at 41–58% moisture.

Our studies show that under the appropriate environmental conditions, *A. flavus* can be a parasite of maize kernels. At the highest temperature tested, 28% of the kernels was infected with *A. flavus*. Field conditions favorable for infection by *A. flavus* occur in certain years in the southern U.S.A. In separate studies in North Carolina, Jones et al (14) reported 15.9% and Marsh and Payne (20) reported 27% of maize kernels from inoculated ears infected with *A. flavus*. In the absence of inoculation, the percentage of infection appears to be around 4% (13,17,20). Even a low percentage of kernels infected with *A. flavus* is economically important. One infected kernel may contain 500,000 ppb aflatoxin (5), 25,000 times the level allowed by the U.S. Food and Drug Administration. Therefore, even a few infected kernels can cause significant contamination of maize.

In our study we rarely observed sporulation from infected kernels while they were on the ear. Sporulation of the fungus in the field is most often associated with injured kernels, and a relationship between aflatoxin contamination and insect injury has been shown. Although insects are definitely involved in the contamination of maize with aflatoxin, we think direct infection by *A. flavus* has a significant role in the epidemiology of this disease, and high temperature appears to be one of the critical factors affecting the infection process.

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