Concurrent Infection of Individual Corn Kernels with White and Green Isolates of *Aspergillus flavus*

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ABSTRACT

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When ears of maize (*Zea mays*) were needle-inoculated in the field with a white conidial isolate of *Aspergillus flavus* at 2 days after midsilk, then reinoculated 4 days later with a green conidial isolate of the fungus, both isolates could be recovered from individual kernels. Both isolates also were recovered from individual kernels from reciprocally inoculated ears. Percentage of kernel infection with the green conidial isolate was higher overall than that with the white isolate in tests over a 3-yr period. Inoculation of ears first with the white isolate, then with the green isolate reduced the amount of infection obtained with the green isolate, whereas inoculation first with the green isolate, then with the white isolate did not influence the amount of infection obtained with the green isolate. Time of inoculation with the green isolate did not influence kernel infection with the white isolate. Percentage of infection was higher after inoculation with both isolates than after inoculation with a single isolate. Individual isolates or both isolates could be recovered from the same kernel or from separate, transversely cut kernel segments. The white isolate was recovered from significantly more center kernel segments than from other segments. Recovery of both fungal isolates from segments from most regions of the kernel supports earlier findings that *A. flavus* infects kernels mainly through the pericarp.

Infection in maize (*Zea mays* L.) by *Aspergillus flavus* Link:Fr. and subsequent aflatoxin contamination is a serious problem in the southeastern United States (1,2,15). The fungus causes little, if any, measurable loss in corn yields directly (12). The magnitude of the *Aspergillus*/aflatoxin problem generally becomes apparent only when the effects of feeding grain contaminated with high levels of aflatoxin to livestock and poultry are observed (1,15). The United States Food and Drug Administration prohibits interstate shipment of corn containing more than 20 ppb of aflatoxin. *A. flavus* infection and aflatoxin contamination also reduce the quality of peanut (*Arachis hypogaea* L.) and cottonseed (*Gossypium hirsutum* L.) products (4,6,8,9).

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The variability of *A. flavus* is well documented (3–9,14,16). Numerous virulent and avirulent and toxigenic and nontoxigenic isolates of the fungus have been identified (4,5,7,14). Cotty (4) reported that simultaneous inoculation of wounded cotton bolls with toxigenic and nontoxigenic strains of *A. flavus* resulted in reduced aflatoxin contamination in cottonseed; he did not attempt to recover and identify strains of the fungus from infected bolls. Wilson et al (17) inoculated maize ears with several color mutants of *A. flavus* and one of *A. parasiticus* Speare and found that the isolate of *A. parasiticus* seemed to be more aggressive and survived better than mutants of *A. flavus*.

Zummo and Scott (18) developed a method for inoculating maize ears with *A. flavus* that apparently does not injure kernels and may be used to select for resistance to kernel infection or to compare aggressiveness of *Aspergillus* species or isolates on uninjured maize kernels (20). A dye marker used in the needle inoculations showed that over 50% of kernels on each ear came in direct contact with inoculum. Zummo and Scott (18) reported that sometimes in needle-inoculated ears, *A. flavus* grew and sporulated profusely on silk residues and kernel surfaces inside the husks, but few of the kernels were actually colonized. Although the fungus covered more than 25% of the surface area of some ears, fewer than 4% of the kernels were invaded. If *A. flavus* enters the corn ear through the silk channel, as has been reported for other fungi (12), it can apparently subsist on senescent silks within the husks (10) and may invade

kernels directly through the pericarp, through weak spots in the seed coat, or through the pedicel sometime before harvest. Zummo and Scott (19) reported that a high percentage (84%) of cobs from ears inoculated with *A. flavus* or from uninoculated ears were infected by the fungus but that the correlation of cob infection with kernel infection was relatively low. They concluded that *A. flavus* invades maize kernels mainly through the pericarp.

In 1985, a white conidial isolate, an apparent mutation, of *A. flavus* was recovered from maize kernels at Starkville, Mississippi. When needle-inoculated into maize ears (18), the isolate infected undamaged kernels and produced aflatoxin. The isolate is stable in culture and can be recognized by a characteristic ivory yellow colony color (13), medium colony height, relatively large conidial heads on Czapek solution agar amended with 7.5% NaCl (CSA-S), and production of kojic acid in infected kernels. The isolate has been placed in the Fungus Culture Collection at the USDA Northern Regional Research Laboratory as accession NRRL 20521.

Kernel infection with the white isolate, using needle (18) and pinbar (11) inoculation methods, was generally less than with NRRL 3357, a green, virulent conidial isolate of *A. flavus* (*unpublished*). NRRL 3357 could be distinguished from other green isolates of *A. flavus* by its medium-low colony growth, characteristic olive yellow (13) colony with a white mycelial fringe, morphology of conidial heads on CSA-S, and production of kojic acid in infected kernels. When grown together in culture, the white isolate initially grew slower than NRRL 3357 but eventually equaled it in growth over a 7-day period. Documentation is lacking concerning how strains or isolates of *A. flavus* interact during the invasion of maize kernels.

The objectives of this study were to use maize ears cross-inoculated with white and green isolates of *A. flavus* to determine: 1) what effect inoculation of ears with one fungal isolate before introduction of a second isolate would have on kernel infection by the second isolate; 2) if individual kernels could become infected with both fungal isolates; 3) if the fungus could enter the ear through the shank or base of the cob and infect uninjured kernels; and 4) what areas of the kernel surface are most susceptible to infection with either or both fungal isolates. Ears were inoculated at different times and/or locations in order to allow the first fungal isolate inoculated to become established in the ear and initiate kernel infection before introducing the second isolate.

MATERIALS AND METHODS

Field plots, harvesting, and sampling methods. Maize for this study was grown in replicated single-row plots at the Plant Science Research Center, Mississippi State University, Starkville. Plots were 5 m long and spaced 1 m apart and were overseeded and thinned to 20 plants spaced about 25 cm apart. Only the top ear of each plant was hand-harvested at 60 days after midsilk. Ears with obvious severe bird or insect damage were discarded. Immediately after harvest, ears were dried at 42 C for 7 days in a forced-air dryer, then shelled. Kernels from each plot were stored in paper bags at 6 C and 45% relative humidity until assayed.

Inoculum sources and production and inoculation techniques. Inoculum was produced from a stable culture of the white isolate or from a lyophilized culture of NRRL 3357 obtained from D. T. Wicklow (Northern Regional Research Center, Peoria, IL). Cultures were grown for 14 days in 500-ml Erlenmeyer flasks containing 50 g of corncob grits (Grit-O-Cobs, Maumee, OH) and 100 ml of H_2O. Conidia were washed from the surface of the grits with sterile distilled water containing two drops of Tween 20 per 100 ml. All inoculum for each year's inoculations was prepared at one time and stored at 4 C as an aqueous suspension of 1×10^8 conidia per milliliter. Inoculum was diluted to 2×10^6 on each day of inoculation and kept on ice in the field until used.

Inoculations were made using modifications of the needle inoculation techniques described by Zummo and Scott (18). A tree-marking gun fitted with a 14-gauge hypodermic needle, 35 mm long, with the tip opening plugged and 1-mm holes drilled 6, 8, and 10 mm from the tip was used in all tests. The inoculator delivered 1.7 ml of inoculum containing 1×10^6 conidia per milliliter. Ears were inoculated in: 1) the shank (2 cm below the base of the cob); 2) the base of the cob (2 cm above the base); 3) the lower ear (the needle was inserted at an angle through the husk approximately 5 cm above the base of the ear and the inoculum was injected over the kernels without injury); 4) the top of the ear (the needle was inserted at an angle through the husk approximately 4 cm below the top of the ear and the inoculum was injected over the kernels without injury); and 5) the silk channel (inoculum was injected into the silk channel onto the kernels without injury). The shank, base of the cob, and lower ear inoculations were made 2 days after midsilk, and the other inoculations were made 6 days after midsilk.

Assay. Only undamaged kernels were assayed for incidence of *A. flavus* on CSA-S, which supports growth of *A. flavus* but inhibits growth of bacteria and most other fungi. To eliminate surface microbes, kernels were dipped in 70% ethanol, then submerged in 1.25% NaOCl for 3 min, and rinsed in sterile distilled water. Kernels were plated on CSA-S in 100-mm petri plates (13 kernels per plate) and incubated for 7 days at 28 C. In 1988, 48 kernels from each plot in each test at both planting dates were cut into five pieces approximately 2 mm long with small sterile wire clippers. The pieces were plated serially 10 mm apart (four complete kernels per 100-mm plate) on CSA-S. The plates were incubated for 5 days at 28 C, then examined for fungus growth. Percentage of kernel pieces supporting white or green isolates of *A. flavus* was calculated.

Field experiments. In 1987, to determine what effect inoculation with one isolate before introduction of a second isolate would have on kernel infection by the second isolate, ears of Pioneer Brand 3369A were inoculated at the top (18) on 1, 2, 3, 4, 5, 7, 8, 9, or 10 days after midsilk with 1.7 ml of a conidial suspension of the green fungal isolate. Each ear was also inoculated at the same inoculation site on day 6 after midsilk. Reciprocal inoculations with both fungal isolates were also made. The experiment was conducted with four replications in a split-plot design with fungal isolates as whole plots and inoculation dates as subplots.

In 1988, to identify the inoculation site on the ear that would produce the highest percentage of kernel infection, ears of Pioneer Brand 3369A were inoculated at 2 days after midsilk with 1.7 ml of a conidial suspension of the green fungal isolate in the shank, base of the cob, or lower ear. Each ear was reinoculated 4 days later in the silk channel or top of the ear with the white isolate. Reciprocal inoculations with both fungal isolates were also made. The experiment had six replications in a randomized complete block design and was repeated in a crop planted 30 days after the first one. To determine if individual kernels could become infected with both fungal isolates and to identify areas of the kernel surface where fungal invasion was most frequent, 48 kernels from each plot in both tests were dissected and assayed for fungal incidence.

In 1989, ears of Pioneer Brand 3369A were inoculated in the shank, base of the cob, or lower ear with the green isolate at 2 days after midsilk. Each ear was also inoculated with the white isolate in the silk channel or top of the ear 4 days later. Reciprocal inoculations with both fungal isolates were also made. Ears in some plots were inoculated with only the white or the green isolate in the shank, base of the cob, lower ear, top of the ear, or silk channel. Ears in control plots were not inoculated. The experiment was a randomized complete block design with six replications and was repeated in a crop planted 30 days after the initial planting.

Analysis of data. Plot means for per-

centage of whole kernels or transversely cut kernel parts that were infected with white or green isolates of *A. flavus* were subjected to an analysis of variance. Mean separation was according to the Student-Newman-Keuls multiple range test.

RESULTS AND DISCUSSION

More kernels were infected overall with the green isolate (19%) than with the white isolate (13%) in ears of Pioneer Brand 3369A inoculated with both isolates in 1987 (Table 1). Inoculation of ears with the white isolate 1, 2, or 3 days before inoculation with the green isolate reduced the amount of infection obtained with the green isolate, whereas inoculation with the white isolate after inoculation with the green isolate had no influence on kernel infection with the green isolate (Table 1). Time of inoculation with the green isolate had no detectable influence on kernel infection by the white isolate.

Kernel infection was 23% with the green isolate and less than 1% with the white isolate in ears inoculated with only the green isolate at day 6 after midsilk. Kernel infection with the green isolate averaged 17% when ears were inoculated with the green isolate on day 6 after midsilk and also inoculated with the white isolate on days 1–5 and 7–10 after midsilk and 20% when ears were inoculated with the green isolate on days 1–5 and 7–10 after midsilk and also inoculated with the white isolate on day 6 after midsilk.

Kernel infection with the white isolate averaged 11% when ears were inoculated with the green isolate on day 6 after midsilk and the white isolate on days 1–5 and 7–10 after midsilk and 14% when ears were inoculated with the green isolate on days 1–5 and 7–10 after midsilk and also inoculated with the white isolate on day 6 after midsilk. Kernel infection was 17% with the white isolate and 4% with the green isolate in ears inoculated with only the white isolate on day 6 after midsilk. Percentages of kernel infection with the green isolate in ears inoculated with the white isolate on day 6 after midsilk and the green isolate on days 1–5 and 7–10 after midsilk did not differ significantly. Percentages of kernel infection with the white isolate in ears inoculated with the green isolate on day 6 after midsilk and with the white isolate on days 1–5 and 7–10 after midsilk did not differ significantly on individual days.

Because the data from the repeat test in 1988 were similar to those in the first test, data from both tests were combined (Table 2). Percentage of kernel infection with the green isolate was significantly higher than with the white isolate. Percentage of kernel infection with the green isolate was similar when ears were inoculated in the silk channel, top of the ear, or base of the ear. Percentage of kernel infection with the white isolate, on the other hand, was highest when ears were inoculated in the silk channel and lowest when inoculated in the shank (Table 2). There was no significant difference in percentage of kernel infection with the green isolate when it was inoculated in the silk channel or top of the ear after the white isolate had been introduced into the shank, base of the cob, or lower ear. Similarly, there was no significant difference in percentage of kernel infection with the white isolate when it was inoculated into the silk channel or top of the ear after the green isolate was inoculated into the shank or base of the ear. The green isolate apparently was able to infect corn kernels when it was inoculated into the shank of the ear, whereas the white isolate showed little apparent ability to infect kernels when it was inoculated into the shank of the ear (Table 2). Percentage of combined infection with both isolates was generally higher when ears were inoculated in the silk channel or top than when ears were inoculated in the shank, base of the cob, or lower ear.

There was no interaction among isolates and ear inoculation sites in 1989. Because data from both tests were similar, results from both experiments were combined (Table 3). There was no significant difference in percentage of kernel infection with either isolate, whether ears were inoculated only once with a single isolate or inoculated with both isolates (Table 3). Percentage of combined kernel infection was higher when ears were inoculated at different times with each of the isolates than when ears were inoculated with only a single isolate. Time of inoculation with either isolate had no influence on kernel infection by the other isolate.

When kernels were cut transversely into five segments and assayed in 1988, the green isolate was recovered from 11.2% of kernel segments, the white isolate from 3.1%, and both isolates from 1.0% (Table 4). In an earlier test with both conidial isolates in 1987, the green isolate was recovered from 21.5% of kernel segments, the white isolate from 4.0%, and both isolates from 6.0% (*data not shown*). Neither isolate was recovered from the silk scars of kernels.

Table 1. Percentage of kernels infected with green and/or white isolates of *Aspergillus flavus* from replicated plots of Pioneer Brand 3369A corn inoculated with both isolates of the fungus in 1987

Time of inoculation after midsilk		Infection with green isolate	Time of inoculation after midsilk		Infection with white isolate
White	Green	(%)	Green	White	(%)
1 day	6 days	14 bc[z]	1 day	6 days	12 a
2 days	6 days	21 a–c	2 days	6 days	13 a
3 days	6 days	10 c	3 days	6 days	15 a
4 days	6 days	10 c	4 days	6 days	19 a
5 days	6 days	11 c	5 days	6 days	8 a
...	6 days	23 ab	...	6 days	17 a
7 days	6 days	28 a	7 days	6 days	11 a
8 days	6 days	15 bc	8 days	6 days	11 a
9 days	6 days	24 ab	9 days	6 days	14 a
10 days	6 days	14 bc	10 days	6 days	19 a
Uninoculated		4			1

[z] Each value is the mean percentage of kernels infected with green or white isolates of *A. flavus* in four replications of 390 kernels per replicate for each inoculation day after surface sterilization and 7 days of incubation at 28 C on Czapek solution agar amended with 7.5% NaCl. Values in a column not followed by the same letter differ significantly ($P = 0.05$) according to the Student-Newman-Keuls multiple range test.

Table 2. Percentage of kernels infected with green and/or white isolates of *Aspergillus flavus* from Pioneer Brand 3369A corn inoculated with both isolates of the fungus in 1988

	Infection (%)	
Isolate/ear area inoculated	Green	White
White/shank, green/silk channel	19.4 ab[z]	0.7 c
Green/shank, white/silk channel	17.2 a–c	10.9 a
White/top of ear, green/base of cob	13.0 bc	8.8 ab
Green/top of ear, white/base of cob	26.8 a	4.9 b
White/shank, green/top of ear	19.0 a–c	0.5 c
Green/shank, white/top of ear	9.4 c	7.4 ab
White/lower ear, green/silk channel	24.9 a	4.7 b
Green/base of cob, white/silk channel	18.2 a–c	10.5 a

[z] Each value is the mean percentage of kernels infected with green and/or white isolates of *A. flavus* in 12 replications of 390 kernels for each inoculation treatment after surface sterilization and 7 days of incubation at 28 C on Czapek solution agar amended with 7.5% NaCl. Values in a column not followed by the same letter differ significantly ($P = 0.05$) according to the Student-Newman-Keuls multiple range test.

Table 3. Percentage of kernels infected with green and/or white isolates of *Aspergillus flavus* from Pioneer Brand 3369A corn inoculated with both isolates of the fungus in 1989

Isolate/ear area inoculated	Infection (%)	
	Green	White
White/shank, green/silk channel	7.7 a–c[z]	1.0 b
White/silk channel, green/shank	2.5 cd	3.6 ab
White/base of cob, green/silk channel	10.7 ab	4.8 ab
White/silk channel, green/base of cob	11.1 a	5.5 a
White/lower ear, green/top of ear	6.3 a–d	1.1 b
White/top of ear, green/lower ear	3.6 cd	3.7 ab
White/shank	···	0.4 b
White/silk channel	···	6.6 a
White/base of cob	···	2.4 ab
White/lower ear	···	0.7 b
White/top of ear	···	6.3 a
Green/silk channel	7.5 a–c	···
Green/shank	3.3 cd	···
Green/base of cob	9.8 ab	···
Green/lower ear	4.9 b–d	···
Green/top of ear	4.9 b–d	···
Uninoculated	0.9 d	0.2 b

[z]Each value is the mean percentage of kernels infected with green and/or white isolates of *A. flavus* in 12 replications of 390 kernels for each inoculation treatment after surface sterilization and 7 days of incubation at 28 C on Czapek solution agar amended with 7.5% NaCl. Values in a column not followed by the same letter differ significantly ($P = 0.05$) according to the Student-Newman-Keuls multiple range test.

Table 4. Percentage of transversely cut kernel segments infected with green and/or white isolates of *Aspergillus flavus* from combined treatments of Pioneer Brand 3369A inoculated with both isolates of the fungus in 1988

Kernel segment	Isolate recovered		
	Green	White	Both
Apical end	12.5 a[z]	3.1 b	1.0 a
Upper intermediate	12.8 a	3.5 b	1.0 a
Center	13.3 a	4.2 a	1.2 a
Lower intermediate	9.7 b	3.5 b	1.0 a
Pedicel end	7.7 c	2.5 b	0.7 a

[z]Each value is the mean percentage of kernel segments infected with green and/or white isolates of *A. flavus* in 12 replications of 48 transversely cut kernels after surface sterilization and 5 days of incubation at 28 C on Czapek solution agar amended with 7.5% NaCl. Values in a column not followed by the same letter differ significantly ($P = 0.05$) according to the Student-Newman-Keuls multiple range test.

The green isolate was recovered from significantly more center or upper segments of kernels than from lower or pedicel segments. The white isolate was recovered from significantly more center kernel segments than from other segments. The recovery of both fungal isolates from kernel segments from most regions of the kernel supports earlier findings that kernel infection by *A. flavus* occurs mainly through the pericarp. It is likely, however, that cracks, breaks, or weak spots in the pericarp may further predispose kernels to infection by *A. flavus*.

These tests show that individual kernels on corn ears inoculated with two distinct isolates of *A. flavus* can become infected with both isolates of the fungus. Individual isolates could be recovered from separate transversely cut segments of a kernel or both isolates could be recovered from the same kernel segment. The recovery of one or both fungal isolates from kernel segments from widely different areas of the kernel surface indicates that *A. flavus* invades maize kernels through the pericarp.

Inoculation of ears with one isolate before the second isolate was introduced did not detectably inhibit kernel infection by the second isolate in 1988 and 1989. Time of inoculation of ears with the green isolate did not influence kernel infection by the white isolate. Percentage of infection was generally higher in ears inoculated with both isolates than in those inoculated with only a single isolate. Percentage of infection was generally higher when both isolates were inoculated in the silk channel or top of the ear than when inoculated in the shank or the base of the cob.

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