Identification and Effects of Maize Silk Volatiles on Cultures of *Aspergillus flavus*

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Volatiles generated from corn silks of individual genotypes of maize were found to exhibit differences in biological activities when the volatiles were exposed to 5-day solid cultures of *Aspergillus flavus*. In inverted potato dextrose−agar Petri plate bioassays, it was found that volatiles emitted from silks of the different maize genotypes had a profound effect on the growth of the fungus and, consequently, aflatoxin production. To determine the underlying cause for this bioactivity, volatiles emitted from the maize silks were trapped on Tenax glass columns and were analyzed by GC-MS. Aflatoxin field-resistant maize genotypes exhibited a larger relative concentration of the antifungal aldehyde, furfural (2-furancarboxaldehyde), when compared to the relative concentrations of the field-susceptible varieties tested. In a closed-container 5-day study, it was observed that fresh 1- and 4-day-old corn silk samples of aflatoxin-resistant maize genotypes emitted higher concentrations of furfural compared to those from susceptible genotypes. This observation probably explains the reason for the bioactivity observed in the in vitro bioassays, and the presence of furfural appears to contribute to a defense mechanism for protecting the developing maize kernel from fungal attack.

Keywords: *Maize (Zea mays); cornsilk; Aspergillus flavus; aflatoxin; volatiles*

INTRODUCTION

Aflatoxins are polyketide-derived secondary metabolites produced by the imperfect fungi *Aspergillus flavus* (Fries) Link and *Aspergillus parasiticus* Speare (Bennett and Chistensen, 1983). The fungal metabolites, especially aflatoxin B_1, are toxic to both humans and animals and are among the most carcinogenic of all natural compounds (Busby and Wogan, 1979; Groopman et al., 1981). Aflatoxin contamination of corn (*Zea mays* L.) and other important food crops continues to be a major economic and health problem, especially in the southwestern United States. Our research is aimed at correlating differences in susceptibility to *A. flavus* and aflatoxin contamination with differences in the chemical makeup of various susceptible and resistant genotypes. Recently, we have described antifungal volatiles from ground maize kernels from different maize genotypes with resistance to *A. flavus* and aflatoxin formation (Zeringue et al., 1996). We recently have also described a correlation between volatile chemicals released from extracted kernels from different positions of the maize ear and *A. flavus* susceptibility and aflatoxin formation (Zeringue, 1997).

The volatile oil components resulting from steam distillations of corn tassels (Buttery et al., 1980), corn kernels and husks (Buttery et al., 1978), and corn silks (Flath et al., 1978) by investigators searching for possible corn ear worm (*Heliothis zea* Boddie) attractants have been reported. These investigators used steam distillation procedures that involved heating the varied maize tissues to extract the volatile oils before volatile determinations were made. In this study, we found differences in biological activity when solid cultures (incubated at 30 °C) of aflatoxigenic *A. flavus* were exposed to volatiles emitted from silks of maize genotypes with different susceptibilities to *A. flavus* infection. The purpose of this study was to identify the volatiles emitted at 30 °C (the same temperature at which we found differences in biological activity) and attempt to determine which volatiles might be responsible for the bioactivity observed.

MATERIALS AND METHODS

Fungal Culture and Spore Suspension. A wild-type aflatoxigenic isolate of *A. flavus* (SRRC-1000A) obtained from Arizona field cotton was cultured on potato dextrose−agar (PDA) Petri plates. After 7 days of incubation at 28−30 °C, spores were harvested and a spore suspension (2.1×10^4 spores/mL) of inoculum in deionized water containing 1% Triton X-100 was used as inoculum.

Sources of Maize Genotypes and Authentic Standards. Several varieties of maize were studied. GT-MAS:gk (Widstrom et al., 1987; Brown et al., 1993) was obtained from the USDA/ARS, Insect Biology and Population Management Laboratory, in Tifton, GA. Pioneer Hybrid 3136 (lot P96PA) was grown in our test field at the SRRC in 1997. Yellow Creole and Huffman were field tested by Zuber et al. (1983) and were obtained from same. Yellow Sweet Jubilee Hybrid seeds were obtained from a local retail market. All genotypes tested were grown in our experimental fields at the SRRC in 1997, and maize ears were all harvested at the same silk emergence stage. No insecticide or other sprays were used during the growing period. To obtain representative samples, the silk bundles from five different plants of each genotype were combined, weighed, placed in polyethylene bags, and frozen at −20 °C until tested and analyzed for volatile components. Authentic standards were obtained from Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI); 2-furancarboxaldehyde (furfural) was obtained from Fluka (Ronkonkoma, NY).

Volatile Assay. Whole, folded fresh cornsilks (0.5, 1.0, or 1.5 g) were placed in an inverted lid of a 100 mm × 5 mm Petri plate set. A sterile single layer of sterilized cheesecloth was placed over the silks to hold them in place. Eighteen milliliters (15.4 g) of PDA was applied to the bottom plate of

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Table 1. Effects on Radial Growth (RG) and Aflatoxin Production (AP) of 5-Day Cultures of Aflatoxigenic *A. flavus* (SRRC 1000A) Exposed to Volatiles from 0.5, 1.0, and 1.5 g of Maize Silks from Five Different Genotypes of Maize

	% of control					
	0.5 g		1.0 g		1.5 g	
genotype	RG	AP	RG	AP	RG	AP
Pioneer 3136 (S)[a]	96 ± 1.2[b]	39 ± 4.8	93 ± 2.1	39 ± 3.3	94 ± 2.3	28 ± 4.6
GT-MAS:gk (R)[a]	85 ± 0.8	20 ± 3.4	78 ± 1.6	68 ± 4.5	87 ± 2.6	55 ± 5.2
Yellow Creole (R)	81 ± 1.3	35 ± 4.1	77 ± 0.9	22 ± 3.6	72 ± 1.4	18 ± 4.3
Huffman (S)	98 ± 1.7	94 ± 3.0	96 ± 2.4	90 ± 4.0	95 ± 2.8	92 ± 5.2
Yellow Sweet Jubilee Hybrid	91 ± 2.2	88 ± 2.4	86 ± 1.8	84 ± 3.5	50 ± 0.6	43 ± 4.1

[a] (R), aflatoxin resistant; (S), aflatoxin susceptible. [b] Values are the means ± standard deviation of three replicates. Radial growth control = 64.6 ± 0.8 mm (n = 15). Aflatoxin B_1 control = 6345 ± 299 ng/g PDA (n = 15).

the Petri plate set; a 10 mm agar plug was extracted with a sterile cork borer from the center section of the solidified PDA. Fifty microliters of *A. flavus* (SRRC 1000A) spore suspension (2.1×10^4 spores/mL) was placed in the center of the 10 mm extracted center well of the PDA. The bottom of the Petri plate set (containing the *A. flavus* inoculated PDA) was then placed over the inverted top containing the silks of the maize genotypes to be tested. The two halves of the Petri plates were sealed with Parafilm. Control plates were constructed in the same way except no silks were added. There were three replicates of each tested sample level. After 5 days of incubation at 30 ± 0.2 °C, radial growth and aflatoxin production were determined (Zeringue and McCormick, 1990).

Trapping and Purging of Volatiles and GC-MS Methods. A 25 mg sample of maize silks from each genotype tested was placed in a glass tube (84 mm × 9 mm, 1 mm i.d.), and glass wool plugs were inserted into each end of the glass column to contain the silks. The tube was placed in an external closed inlet device (ECID) (Legendre et al., 1979), and the tube was purged at 30 °C with a helium flow of 55 mL/min for 1 h. Volatiles from the silks-containing tube were purged into a similar glass tube containing 0.1 g of Tenax GC (60/80 mesh), which was also plugged with glass wool at both ends. The glass tube containing the Tenax GC and any trapped volatiles was then placed into the ECID, and the volatiles were desorbed at 135 °C at a helium flow of 20 mL/min for 3 min onto a capillary gas chromatographic column (HP-5, cross-linked 5% phenyl methyl silicon column, 50 m × 0.2 mm, 0.5 μm film thickness). The GC oven temperature (contained in an HP 5890A-5971A GC-MS system or an HP 5890 series II-5989A GC-MS system) was cryogenically lowered to −30 °C for 3 min and was held at this initial temperature during the heat desorption stage. The GC oven was programmed to 30 °C at a rate of 5 °C/min and then to 250 °C at a rate of 15 °C/min; the temperature was held at 250 °C for 5 min. Electron ionization was operated at 70 eV, helium linear velocity was set at 30 cm/s, and the internal injector was adjusted at 150 °C. Identification of separated components was based on comparison of their retention time and mass spectra to those of authentic samples and by computer matching of the unknown spectra with reference mass spectra in the HP G1035A AA9 Wiley 130 K mass spectral databases (Wiley, 1986). Quantitation was accomplished by use of external authentic standards and the HP 1030A computer ChemStation.

In a 5-day study of furfural emitted from corn silks, 25 mg maize silk samples from each genotype were placed in glass columns as above. Each glass column containing silks was placed into a Teflon-lined screw type test tube, capped, and stored at 30 °C; volatiles from the silks were trapped on Tenax GC and purged as above. Three replicates of each sample were obtained.

RESULTS AND DISCUSSION

Corn silk volatiles from all of the maize genotypes tested exhibited some change on both radial growth and aflatoxin production of aflatoxigenic *A. flavus* (Table 1). Volatiles from resistant GT-MAS:gk silks resulted in the greater aflatoxin inhibition at the lowest concentration of maize silks tested (0.5 g) compared with other genotypes tested, but field-resistant Yellow Creole corn silks at the highest concentration of silks tested (1.5 g) resulted in the lowest aflatoxin amounts overall. Volatiles from aflatoxin-susceptible Pioneer 3136 resulted in little effect on growth (93−96% of control) but exhibited a noticeable decrease in aflatoxin formation (28−39% of control). It is possible that a volatile or a combination of volatiles liberated from the silks of Pioneer 3136 inhibited the aflatoxin pathway.

The maize silk volatiles, which had been collected on Tenax glass columns at 30 °C and subsequently separated and identified by GC-MS, are shown in Table 2. Figure 1, the total ion chromatogram of the aflatoxin field-resistant Yellow Creole genotype, is shown as a representative silk volatiles chromatographic run. Each separated compound identified in this particular run (Figure 1) is represented by a number both in Figure 1 and in Table 2. The relative concentrations (based on measurement of peak areas) of the separated components of the maize silks are presented in Table 2. Forty-eight volatile compounds were identified from the five maize genotypes. The major classes of compounds found included alcohols, aldehydes, ketones, furans, and organic acids. 2,3-Butanediol was found in four of the five genotypes at relative concentrations (RC) from 3.0 to 5.7%. Furfural, hexanol, heptanol, and 2-methylbutanal were the major aldehydes identified. Alkanals and alkenals are reported to have strong fungicidal properties against aflatoxigenic *A. flavus* (Zeringue and McCormick, 1990; Hamilton-Kemp et al., 1992). A 5-day incubation assay with varied amounts of hexanal (Zeringue et al., 1996) showed that 83 μg of hexanal completely inhibited radial growth (RG) and aflatoxin production (AP) of aflatoxigenic *A. flavus*, 8.3 μg of hexanal inhibited RG by 20% and AP by 34%, and 2.1 μg of hexanal inhibited RG by 11% and AP by 17%.

Furfural was the major aldehyde produced by aflatoxin field-resistant genotypes of maize (Table 2); 14.5% RC of the silk volatiles emitted by Yellow Creole and 17.5% RC of the volatiles emitted by GT-MAS:gk consisted of furfural. Lesser amounts of furfural were emitted by silk volatiles from the two susceptible genotypes (Huffman and Pioneer 3136), which were 5.8% RC and 5.3% RC, respectively. Recently we found in a 5-day gaseous culture bioassay (Zeringue, 1997) that 0.12 μg of furfural inhibited RG by 28% and AP by 31%; 3.5 μg of furfural completely inhibited RG and AP. Furfural occurs naturally in the maize ear and apparently is a normal breakdown product from pentosans contained in the silks (Jones et al., 1980).

Table 3 gives the results of a 5-day study of the emission of furfural from corn silks in closed containers. Note that the field-resistant genotypes emitted 2−3

Table 2. Volatile Maize Silk Components

		maize genotypes[a]				
	RT[b]	YC	HUFF	MAS	3136	Y/SW
alcohols						
2,3-butanediol	27.37		3.9	4.8	3.0	5.7
ethanol	14.18		1.6	3.9	3.6	3.7
1,2-propandiol (9)[c]	23.24	0.3	0.1	0.2		0.5
2-furanmethanol (13)	27.19	0.6	0.3	0.4	0.4	0.6
ketones						
2,3-butanedione	21.97		1.4	3.5	2.8	4.1
3-hydroxy-2-butanone	25.81		0.5	1.2	1.1	3.8
3-methyl-2,5-furandione (17)	30.40	0.5	0.5	0.4	0.1	0.3
dihydro-2(3*H*)-furanone (16)	29.35	0.7	0.4	0.7	0.2	0.2
2-heptanone (14)	28.10	0.2	1.7	0.2	0.3	0.3
3-octanone (29)	40.81	0.5	0.1		0.1	2.2
(*E*)-3-octen-2-one (22)	33.88	0.9	0.6	0.2	2.2	0.2
1-hydroxy-2-propanone (8)	21.33	2.3	2.7		0.6	0.6
2,4-pentanedione	24.96		0.3		0.6	1.3
2,3-dihydro-3,5-dihydro-4*H*-pyran-4-one (26)	37.95	25.7	14.5	13.4	7.5	10.7
aldehydes						
benzeneacetaldehyde (23)	34.21	0.9	1.7	0.4	0.1	0.1
2-methylbutanal (5)	17.98	8.8	7.3	3.2	6.2	4.5
decanal (27)	39.60	0.2	2.2	0.4	0.5	2.6
(*E*,*E*)-2,4-decadienal (31)	42.09	0.4	0.3	1.2		3.3
trans,*trans*-nona-2,4-dienal (32)	42.66	0.8			0.4	
(*Z*)-2-decenal	43.69			0.5	0.3	1.4
heptanal (15)	28.54	2.6	3.3	8.7	2.1	2.3
hexanal (10)	24.22	6.3	5.0	5.9	5.0	10.7
(*Z*)-2-heptenal (30)	41.23	0.5	0.5		0.4	4.3
(*E*)-2-octenal	34.59		0.3	2.2	0.4	1.8
propanal	17.92		3.6		3.4	3.4
2-methylpropanal (3, 4)	13.06	3.8	2.2	3.4	1.8	3.3
pentanal (6)	19.55	0.5	1.9	2.7	1.6	1.9
nonanal (25)	36.21	0.4	0.3	0.1	0.1	0.2
2-undecenal (33)	43.68	0.5	0.3	0.3	0.3	
5-(hydroxymethyl)-2-furancarboxyaldehyde	40.31		2.8	1.2	3.2	4.2
furfural (11)	25.70	14.5	5.8	17.5	5.3	6.2
furans, acids						
furan (18)	30.77	1.8	2.1	0.2	5.3	0.7
2-pentylfuran (21)	32.15	2.1	1.0	0.9	1.1	0.7
2,3-dihydrobenzenfuran (28)	39.92	0.2		0.1	0.2	
2-hexylfuran	42.66		1.7	2.6	4.2	0.2
acetic acid (7)	20.61	16.4	11.5	8.4	16.3	7.2
4-(2-methoxy-1-methyl-2-butanoic acid (20)	31.81	1.3		0.6		
hexanoic acid (19)	31.60	1.4	2.6		2.8	
miscellaneous						
benzene	24.45				1.2	
diethyl-*o*-phthalate ethyl acetate (34)	48.16	0.7	0.2	0.2	0.4	0.6
dotriacotane	33.08					0.6
acetic acid, ethyl ester	24.86		3.6	3.4	4.9	2.6
hexane	21.75		1.6		0.8	
dichloromethane	20.50		3.9	5.4	5.3	
sulfinylbis(methane) (12)	26.34	1.4	4.0	1.0	3.3	
thiobis(methane) (1, 2)	10.69	2.8	0.3			
nonane (24)	34.57		1.4	0.6	0.4	

[a] YC, Yellow Creole; HUFF, Huffman; MAS, GT-MAS:gk; 3136, Pioneer Hybrid 3136; Y/SW, Yellow Sweet Jubilee Hybrid. [b] Retention time. [c] Peak numbers corresponding to the peaks in Figure 1.

times more furfural in 1-day-old silks than the field susceptible varieties and that the 4-day-old silks of resistant genotypes emitted 4−12 times more furfural than susceptible types.

The ketone class of compounds was dominated by 2,3-dihydro-3,5-dihydro-4*H*-pyran-4-one (DDMP). The RC of this compound compared among the different genotypes ranged from 7.5 to 2.5%. DDMP could possibly result enzymatically from a reaction of either fructose or glucose with protein or amino acids through a Heyns rearrangement (Nishibori and Kawakishi, 1994). Under nonenzymatic conditions, this reaction usually requires heat (150 °C) to take place, but it apparently forms under the incubation culture conditions enzymatically. DDMP saponin is widely distributed in leguminous seeds and has been shown to exhibit active oxygen scavenging activity (Yoshiki and Okubo, 1995). At this time, it is uncertain if DDMP is involved in microbial defensive strategies in cotton and corn. Furan and several substituted furans were identified; furan could result from decarboxylation of furfural, and 2-pentylfuran has been reported by others from the volatile oil of maize silks (Flath et al., 1978; Buttery et al., 1978).

Acetic acid ranged from 7.2 to 16.4% RC; the ethyl ester of acetic acid and hexanoic acid were predominant in the organic acid class (Table 2). Furfural breakdown could lead to acetic acid (Rivard and Grohmann, 1991). Diethyl-*o*-phthalate ethyl acetate (miscellaneous, Table 2) probably is a contaminant because of its widespread use as a plasticizer. Dichloromethane, sulfinylbis(methane), and thiobis(methane) were found in 0.3−5.4% RC. Furfural breakdown can result in its final conversion to methane and carbon dioxide (Rivard and Grohmann, 1991).

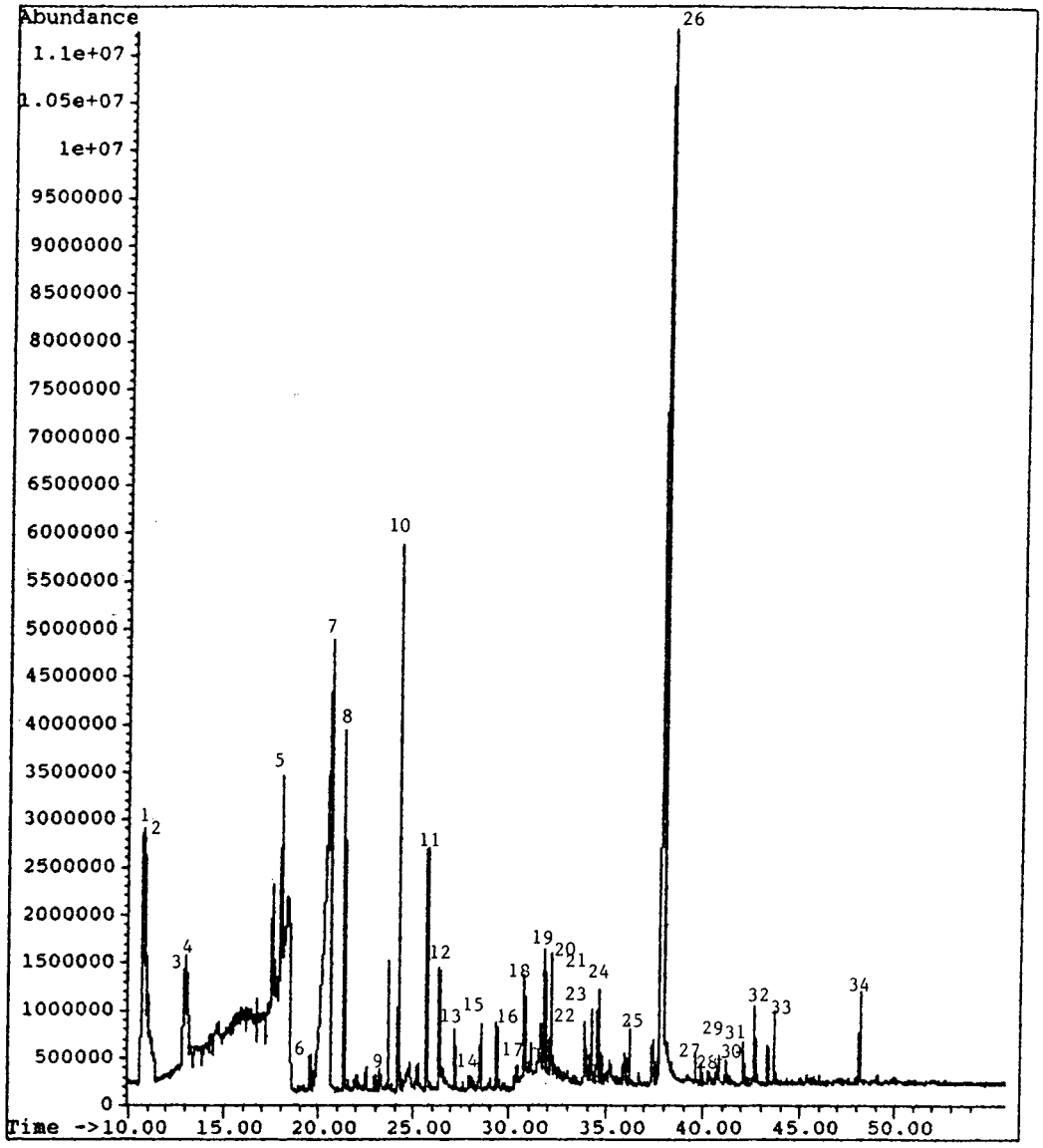

Figure 1. Total ion chromatogram of maize silk volatiles of aflatoxin field resistant Yellow Creole genotype. For GLC-MS conditions, see text. Identity of peaks is given in Table 2.

Table 3. Furfural Generated from 25 mg of Maize Silks from Five Different Maize Genotypes after 1 and 4 Days at 30 °C in a 5-Day Closed-Container Study

	furfural (ng/h)	
genotype	1-day-old	4-day-old
Yellow Creole (R)[a]	13.2 ± 1.1[b]	28.9 ± 1.9
Huffman (S)[a]	3.1 ± 0.4	2.4 ± 0.2
GT-MAS:gk (R)	10.5 ± 0.5	12.3 ± 1.3
Pioneer 3136 (S)	4.3 ± 0.8	3.8 ± 0.3
Yellow Sweet Jubilee Hybrid	6.9 ± 0.3	5.7 ± 0.2

[a] (R), aflatoxin resistant; (S), aflatoxin susceptible. [b] Values are the means ± standard deviation of three replicates.

In summary, it is likely that furfural is responsible for the antifungal activity observed in the inverted PDA and Petri plate assays. Volatile relative concentrations of furfural range from 2.5 to 3.3 times more in the silks of the aflatoxin-resistant maize varieties. There is also an increased relative concentration of the C_6 and C_7 alkanals in these same resistant genotypes, which also may contribute to the antifungal conditions observed.

The volatiles emitted from cornsilks apparently contribute to an overall defense mechanism that results in protecting developing maize kernels from *A. flavus* infection.

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