Relationships Between Cotton Leaf-Derived Volatiles and Growth of *Aspergillus flavus*

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Microbial-free compressed air was passed continuously for 2- and 7-day test periods through enclosed systems containing wounded or nonwounded leaves of glanded or glandless cotton; the resultant emitted volatiles were bubbled through liquid cultures of *A. flavus*. After two days incubation, volatiles from wounded glanded and wounded glandless cotton leaves retarded the growth of *A. flavus*. After seven days incubation, fungal growth was stimulated in cultures which received volatiles from wounded or nonwounded glanded cotton leaves, but not from either type of glandless cotton leaves. Volatile profiles of the leaves were obtained by GC/MS examinations of the leaves at 2- and 7-day time periods. Purified compounds of selected identified volatiles were assayed with *A. flavus* to determine which volatiles might be responsible for the bioactivity described.

Aspergillus flavus Link infects cottonseed in the field, especially in desert areas of the Southwestern United States (1). Once established within the seed, *A. flavus* synthesizes aflatoxin, a known carcinogen. Aflatoxin contamination of cottonseed therefore reduces the quality and economic value of this crop and presents a major health problem to both animals and man. Our overall research objective is the elimination or reduction of aflatoxin contamination in cottonseed through the enhancement of natural resistance factors already present in cotton.

Vast amounts of literature exist on the ability of plants to produce secondary metabolites to utilize in defense mechanisms, but little is known about how volatile compounds produced by plants fit into these defense strategies. Phytoalexins are examples of secondary metabolites produced by plants that hinder the invasion of microorganisms in the host-plant tissues. It is known that some plants are capable of producing volatile compounds that have very effective antimicrobial activities, e.g. mustard oils (*Cruciferae*), garlic extract (*Allium sativum*) and hydrocyanic acid (Sorbus species) (2,3). These volatile, bioactive compounds induced by invading microoganisms may be said to function as "gaseous phytoalexins."

Here we report the head-space volatiles derived from wounded (W) and nonwounded (NW) glanded (SJ-2) and glandless (G 8160) cotton leaves and the effects of these naturally occurring compounds on the growth of *A. flavus*.

EXPERIMENTAL PROCEDURES

Plants and treatment of leaves. Third and fourth true leaves from two-month-old postemergence Acala SJ-2 (glanded) and Shafter G 8160 (glandless) cotton plants grown under greenhouse conditions were harvested with attached petioles. Leaf areas and weights were matched as nearly as possible for each experimental run. Detached leaves and attached petioles were surface-sterilized by successive immersions for 30 sec in 20% Clorox, 70% ethanol and sterile distilled H_20. Abraded wounds were produced by gently scraping the underside of each leaf with a scalpel blade. Wounding in this fashion damaged ca. 20% of leaf surface.

Apparatus. In each test, 20 leaves were placed in each of four sterilized 150mm i.d. Wheaton dry seal desiccators. Sterile techniques were used in positioning the leaves within the desiccators. Each of the desiccators contained either (NW) or (W) SJ-2 or G 8160 cotton leaves. The petioles of each leaf were placed through the holes of the porcelain desiccator plate, and the basal cut areas of the petioles were immersed in sterile Hoagland's plant nutrient solution (4). Microbial-filtered compressed air was allowed to flow into the desiccators and exhausted through a microbial filter into a 125-ml Erlenmyer cotton-stoppered flask containing 50 ml Adye and Mateles nutrient media (5) which had been inoculated with 100 μl of an *A. flavus* spore suspension containing 4.8×10^8 spores/ml sterile distilled water. The desiccators received 12 hr illumination/day from a bank of eight 15-watt Plant Gro lights positioned 20 cm above the desiccators. Fungal-inoculated controls received microbial filtered compressed air only and at the same flow rate as the cultures receiving exhaust flow from the desiccators. Separate experimental tests were run continuously for two days or for seven days. No microbial growth was apparent when one ml of used Hoagland's plant nutrient solution resulting from a seven-day experimental run was placed on a potato-dextrose-agar petri plate and incubated for one week.

Collection and idenfitication of the major volatiles emitted from cotton leaves. Volatiles were trapped on small glass Tenax columns (0.1 g Tenax GC 60-80 mesh) porous polymer packed between glass wool in tubes 3/8" × 3"). Air streams were passed over the plant material, and volatiles were collected for 30-min periods. The Tenax tubes were loaded into an external inlet apparatus (Scientific Instrument Service, River Ridge, LA) interfaced with a Finnegan MAT GC/MS 4000 Series instrument (6). The volatiles were heat-desorbed onto a 50-m SE-54 column. The temperature program used was three min from -30°C to 30°C (to trap the volatiles at the head of the column) followed by 30-150°C at 2.5 degrees/min and then 150-250°C at 10 degrees/min. The peaks on the reconstructed ion chromatogram were tentatively identified by comparison with the Finnegan NBS Library and quantitated using the AR1CQ program (Table 2).

Assay of individual volatiles on the radial growth of A. flavus. Two 10-mm holes were removed form 18 ml-containing potato-dextrose-agar 82 mm diameter petri plates, one in the center and one along the margin edge of the plate. A spore suspension of *A. flavus* was placed in the center well, and a one-ml glass beaker was positioned

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TABLE 2.

Relative Percentages of Compounds Collected from Nonwounded (NW) or Wounded (W) Acala SJ 2 (Glanded) and Shafter G 8160 (Glandless) Cotton Leaves

Collected components	NW SJ-2 day 2	NW SJ-2 day 7	W SJ-2 day 2	W SJ-2 day 7	NW 8160 day 2	NW 8160 day 7	W 8160 day 2	W 8160 day 7
acetic acid 1-methyl ethyl ester	0.20	...	...	...	...	...	...	...
ethyl acetate	...	...	19.96	12.13	...	...	...	...
2-butoxy ethanol	17.40	...	...	...	...	...	...	...
2-ethoxylethanol	...	0.37	...	...	...	...	...	...
1,3-propane diol-2-hydroxylmethyl 2-methyl	...	...	0.28	...	...	...	...	...
propanoic acid 2-methyl 3-hydroxyl 2,4,4,-trimethylpentyl ester	...	...	...	...	0.65	0.52	0.16	...
2-propanol 1-(1-methylethoxy)	...	...	...	...	0.70	0.32	...	...
propanoic acid 2-methyl α-, α-, dimethyl-1-(2-hydroxylpentyl)	...	...	...	...	...	...	0.05	...
3-methyl 1-butanol acetate	0.80	...	...	...	...	0.30	0.25	...
3-methyl 2-butanol	0.30	...	...	...	...	...	...	...
1-butanol 4-butoxy	...	...	...	1.90	...	2.40	...	...
2,3-butanedione, monooxime	...	...	...	...	3.06	5,64	...	9.47
2-butanol 3,3-dimethyl	...	...	...	...	0.99	0.77	...	...
2-butanone 3,4-epoxy 3-ethyl	...	...	...	...	0.12	0.93	...	...
2-buten-1-ol 3-methyl acetate	...	...	...	...	...	...	1.44	...
butanoic acid 3-hexyl ester	...	...	...	...	...	...	1.16	...
2-pentenal, 4-methyl	...	...	...	...	...	...	7.40	...
3-pentenal, 4-methyl	3.09	...	...	...	...	...	...	...
4-pentenal, 2-methyl	0.56	...	...	...	...	...	...	...
pentanal 4-methyl 2-propyl	...	...	...	...	0.25	0.52	...	...
1-pentanol 4-methyl 2-propyl	0.31	...	0.14	...	...	...	...	...
4-pentanal 2,2-dimethyl	...	...	0.34	...	...	0.05	...	...
4-penten-2-ol	...	...	...	...	0.52	0.74	...	...
3-methyl pentenal	...	...	...	...	...	0.18	...	1.53
1-pentenal 2,2-dimethyl	...	...	...	...	...	0.07	...	1.86
2-penten-1-ol 2-methyl	...	...	...	...	...	...	1.95	...
4-penten-2-one 3-cyclohexyl	...	...	...	...	...	...	0.04	...
hexanal	...	3.54	4.06	...	0.61	3.68	...	...
2,4-hexanedienal	1.10	...	...	...	...	...	...	...
5-methyl hexanone	0.12	...	...	...	...	...	...	...
2-hexenal	9.62	6.83	...	...	...	...	5.65	...
3-hexen-1-ol (3-hexen-1-ol acetate)	8.12	4.38	1.64	2.50	...	8.34	76.91	...
bicylco [2.2.0] hex-1-(4)-ene	...	...	...	...	...	...	1.29	...
bicyclo [3.1.1] hex-2-ene 2-methyl 5-(-1-methylethyl) [3-thujene]	...	3.61	...	...	...	...	...	...
hexanal 4,4-dimethyl	...	...	...	...	...	0.05	0.56	...
2-hexenal 2-ethyl	1.40	...	0.95	...	40.38	28.78		51.42
2, 5-cyclohexadiene 1,4-dione 2,6,6-bis 1,1-dimethylethyl	...	...	0.44	...	...	...	...	...
cyclohexene 1-methyl-4-(1-methylethenyl) (limonene)	10.07	21.81	0.25	...	1.36	3.23	...	...
cyclohexanone	...	0.75	...	...	...	...	...	...
hexanol 2-ethyl 2-propyl	0.24	...	...	...	...	...	...	...
3-hexen-2-one 3,4-dimethyl	...	...	1.59	...	...	...	...	...
2-hexen-1-ol	...	...	0.92	...	...	...	0.83	...
4-hexenoic acid 3-methyl 2,6-dioxo	0.09	...	...	...	...	...	...	...
1-hexenal 2-ethyl 2-propyl	0.22	...	...	...	...	...	...	...
3-methyl hexanal	...	...	...	...	0.22	0.37	...	...
cyclohexene 4-ethenyl 1,4-dimethyl	...	...	...	...	...	...	...	9.12
hexanal 3,3-dimethyl	...	...	...	...	...	...	0.23	...
hexenoic acid 3-hexenyl ester	...	...	...	...	...	...	0.15	...
heptanal 0-decyl	...	0.25	...	...	...	...	...	...
heptanal	0.19	1.88	1.67	...	...	...	...	...
5-hepten-2-one 6 methyl	...	...	0.42	...	...	...	...	...
bicyclo [3.1.1] heptane 6,6-dimethyl 2-methylene [β-pinene]	0.72	0.77	2.65	1.41	...	...	...	...
3-heptanone	...	...	...	...	0.13	0.28	...	...
bicyclo [3.1.1] hept-2-ene 2,6,6 trimethyl [α-pinene]	...	...	...	8.40	...	...	...	...
1-heptanol 6-methyl	...	...	...	2.02	3.85	4.23	...	...

2-hepten-2-one	...	...	...	...	...	...	0.05	...
2-heptenal	17.40	...	...	38.40	...	2.17	...	1.13
1,5-heptadiene 3,4-diol 2-methyl	...	...	2.26	...	...	...	...	...
2-heptyn-2-ol	...	0.39	...	...	...	...	...	...
bicyclo [2.2.1] heptan-2-ol 2,3,3-trimethyl	0.24	...	...	...	...	...	...	...
7-oxabicyclo [4.1.0] heptane 3-methyl	...	0.63	...	...	...	...	...	0.38
5-heptenal 2,6-dimethyl	...	...	...	...	...	...	0.05	...
1,3,7-octatriene 3,7-dimethyl (ocimene)	2.58	...	11.27	...	...	1.03	0.05	0.35
1.7-ocatadiene-3-one 2-methyl 6-methylene	...	2.27	...	...	...	...	...	...
2-octen-1-ol	...	...	10.84	...	0.98	0.80	...	...
1,6-octadiene 7-methyl 3-methylene (myrcene)	17.70	10.52	24.79	5.59	0.27	1.25	...	...
1-octyn-3-ol 4-ethyl	...	...	...	...	39.35	21.01	...	...
isooctanol	...	...	...	...	1.24	...	...	...
2,3-octanedione	...	...	...	...	...	...	...	0.27
octenone	...	...	...	...	...	...	...	21.19
1-octanol 3,7-dimethyl	...	...	...	...	...	...	...	0.07
nonanal	...	11.95	6.45	2.73	...	...	0.28	2.24
nonanol	1.48	...	...	...	0.41	2.06	...	...
2-nonenal	...	4.08	...	...	...	...	...	...
2,6-nonadienal	...	...	...	...	...	...	0.86	...
2-nonanal	...	...	...	...	...	...	0.08	...
1-decanol	0.38	...	...	...	...	...	...	...
1-decanal	...	16.19	0.42	10.82	...	...	0.38	0.29
1-decyne	0.74	...	1.71	2.73	4.29	8.12	0.18	...
3-decyne-2-ol	...	0.34	...	...	...	...	...	...
2-decenal	...	0.89	...	...	...	...	...	...
1,6,10 dodecatriene 7,11-dimethyl 3-methylene	...	1.73	...	...	...	...	...	...
dodecanal	0.22	3.95	...	...	...	...	...	...
4-carene	0.36	...	0.78	...	0.62	2.16	...	...
monoterpene	...	1.73	...	...	...	...	...	...
benzene methanol, α, α di-methyl	4.35	1.14	6.17	11.37	...	...	...	0.68

in the margin well. One to 10 μl levels of purified volatiles (Aldrich Chemical Co., Milwaukee, WI, and Sigma Chemical Co., St. Louis, Missouri) were placed in this outer margin glass well, then the top lid and bottom of the plates were sealed together with tape. Radial growth of the fungus was recorded after two days incubation (Table 3).

RESULTS AND DISCUSSION

Initially two- and seven-day growth patterns of *A. flavus* in liquid culture receiving volatiles emitted from SJ-2 and G 8160 (W) and (NW) cotton leaves were determined using the apparatus pictured in Figure 1. The results of this initial experiment are shown in Table 1. Note that two days after fungal inoculation SJ-2 (W) and G 8160 (W) both demonstrated a retardation of *A. flavus* growth. Results from a separate experiment show that after a 7-day incubation period volatile derived from SJ-2 (W) or (NW) leaves resulted in a stimulation of *A. flavus* growth. In order to gather further information, a Tenax chromatographic collection tube was positioned in line at the

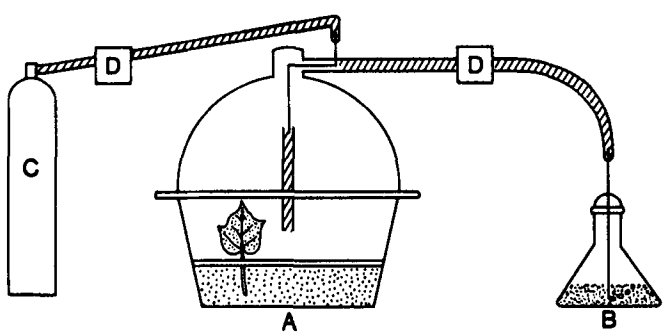

Fig. 1. Cotton leaf volatile emitting apparatus. A. Dry seal desiccator containing leaves with petioles immersed in nutrient solution. B. Erlenmeyer flask containing *flavus* culture. C. Compress air source. D. Microbial filter.

TABLE. 1.

Effects of Two- and Seven-Day Incubation of *A. flavus* in Contact with Cotton Leaf Volatiles

Cotton cultivar	Dry weight as a percent of control
2 days	
8160 NW[a]	90.7 ± 9.1[c]
8160 W[b]	32.4 ± 8.6
SJ-2 NW	115.3 ± 6.7
SJ-2 W	32.4 ± 3.2
7 days	
8160 NW	100.6 ± 3.3
8160 W	96.6 ± 4.0
SJ-2 NW	179.3 ± 5.6
SJ-2 W	178.3 ± 1.9

[a]Nonwounded.
[b]Wounded.
[c]Standard error of mean of 3 separate experiments.

TABLE 3.

Radial Growth of *A. flavus* as a Percent of Control After Two Days in Contact with Some Selected Volatiles.

Volatile component	Level of tested Component (μl)				Concentration
	1	3	5	10	μmol/μl
Alcohols					
3-methyl-1-butanol	91±4[a]	88±3	80±6	55±10	9.1
3-methyl-2-butanol	95±6	95±4	78±3	80±6	9.1
2-buten-1-ol	100±3	100±2	100±3	100±3	1.5
2-butoxy alcohol	96±1	90±1	86±2	86±3	7.6
1-pentanol	109±2	100±3	91±2	90±1	9.2
4-penten-1-ol	98±2	98±6	89±5	89±3	9.6
cis-2-hexene-1-ol	98±2	93±4	98±6	98±3	8.4
cis-3-hexene-1-ol	85±2	80±8	74±5	69±6	8.4
1-heptanol	136±3	114±5	73±3	73±4	7.0
3-hepten-1-ol	100±3	89±2	80±4	73±3	7.0
1-nonanol	136±3	122±3	128±4	132±3	5.7
1-decanol	96±2	92±2	91±2	91±5	5.2
Aldehydes					
hexanal	84±5	76±3	76±2	0±0	8.3
trans-2-hexenal	0±0	0±0	0±0	0±0	8.6
2,4-hexadienal	53±3	0±0	0±0	0±0	9.0
2-hexenal, diethylacetal	98±2	0±0	0±0	0±0	4.9
heptanal	67±5	58±3	49±6	0±0	7.4
trans-2-heptenal	82±3	0±0	0±0	0±0	7.6
octanal	114±7	88±5	50±3	46±3	6.5
trans-2-octenal	77±3	0±0	0±0	0±0	6.7
nonyl aldehyde	75±4	60±3	46±3	0±0	5.8
trans-2-nonenal	82±2	0±0	0±0	0±0	6.0
N-decyl aldehyde	96±2	92±8	91±3	91±3	5.3
dodecyl aldehyde	136±5	112±4	104±3	104±2	4.5
Ketones					
2-pentanone	133±8	116±3	116±3	116±5	9.4
3-pentanone	111±3	121±3	118±2	116±2	9.4
cyclohexanone	109±6	107±5	109±3	91±4	10.0
2-heptanone	80±2	84±2	76±3	71±4	7.1
3-heptanone	100±1	96±5	91±4	91±3	9.6
3-octanone	82±4	82±3	82±1	82±3	6.3
2-nonanone	94±1	77±2	77±2	61±3	5.9
Others					
myrcene	85±5	90±5	88±3	86±3	5.9
ocimene	95±9	95±3	88±1	85±2	5.8
limonene	90±6	85±3	83±3	90±3	6.1
camphene	131±6	111±5	111±3	111±2	6.2
α-pinene	114±5	114±4	114±4	114±1	5.9
β-pinene	114±2	114±3	114±1	114±2	6.3
caryophyllene	93±2	93±2	93±2	93±2	4.4
4-pentenoic acid	114±3	108±2	96±3	86±1	9.8
ethyl acetate	100±4	95±1	91±4	86±3	10.2

[a]Mean ± SD for 3 replicates/tested level.

exhaust end of the volatile-emitting desiccators, which permitted the trapping of the cotton leaf-derived volatiles. Characterization and quantitation of these volatiles resulted in the data presented in Table 2, while the bioassay results describing the growth of *A. flavus* in the gaseous atmosphere containing varied amounts of selected purified volatile components are listed in Table 3.

It is difficult to determine which component in mixtures of volatiles emitted from cotton leaves is responsible for activities influencing fungal growth. It is possible to demonstrate the bioactivity of individual purified components on fungal growth and attempt to relate these results to explain the overall observations. Of the individual volatile components tested, C_6-C_9 alkenals, especially *trans*-2-hexenal, exhibited the most inhibitory effects on the growth of the fungus. *Trans*-2-hexenal is a breakdown product of the unsaturated fatty acids, linoleic and linolenic acids, and its concentration is greatly increased by mechanical damage of tissues (7). The key enzyme for its biosynthesis is a membrane-bound lipoxygenase which is believed to be located both in chloroplasts and mitochondria (8-10). Oxidation of *trans*-2-hexenal results in 2-hexenoic acid, while reduction generates mainly *cis*-3-hexenol ("leaf alcohol") and n-hexanol.

The stimulatory effects of the individual volatile components tested were not as pronounced as the inhibitory effects. These fungal growth stimulatory volatiles exhibited peak activities at the lowest tested concentrations,

and their stimulatory activities were apparent in each of the tested classes of compounds (alcohols, aldehydes, etc.). Unbranched c_8, C_{12}-alkanals, 2- and 3-C_5 alkanones, and the volatile terpenes, α- and β-pinene, stimulated the growth of *A. flavus*.

The bioactivity results of these individually tested volatiles (Table 3) may help to explain the results described in Table 1. Over 90% of the identified volatile components of two-day SJ-2 W and G 8160 W leaves (Table 2) contained volatile components that inhibited *A. flavus* growth; this activity is reported in Table 2. Seven-day SJ-2 W and SJ-2 NW leaves contained minor amounts (less than 10%) of volatile components that were stimulatory (Tables 1 and 2) based on the individually tested component assessment on the growth of *A. flavus* (Table 3). The overall effect, however, was stimulatory even though a larger amount of inhibitory volatiles were present during that collection period. It appears that wounding releases the major volatile components seen in both SJ-2 W and 8160 W at two days, and this induction of an antimicrobial substance may be considered the release of "gaseous phytoalexins." It also appears that the SJ-2 W and SJ-2 NW volatiles that stimulate the fungal growth at seven days are being emitted from the lysigenous pigment glands in the leaves, because this stimulatory effect was not seen in cultures receiving volatiles emitted from glandless G 8160 leaves.

There is interest in manipulating the terpenoid aldehydes such as gossypol and related compounds for resistance in cotton to *Heliothis* species (11). Breeding the cotton plant for elevated levels of linoleic and linolenic acids could result in an enrichment of "gaseous phytoalexins" production induced upon microbial invasion and thus result in increased host plant resistance to *A. flavus* and ultimately to aflatoxin-contaminated cottonseed meal.

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