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CHANGES IN COTTON LEAF CHEMISTRY INDUCED BY VOLATILE ELICITORS

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Key Word Index—*Gossypium hirsutum*; Malvaceae; cotton; *Aspergillus flavus*; volatiles; phytoalexins; heliocides; myrcene; monoterpene; hemigossypolone.

Abstract—Cotton (*Gossypium hirsutum*) leaves were exposed for 7 days to volatile chemicals originating from *Aspergillus flavus*-infected cotton leaves, *A. flavus* cultures or mechanically damaged cotton leaves. Volatiles from *A. flavus*-infected leaves triggered significant increases of 52 and 34% in phloroglucinol-reactive compounds in wounded or undamaged cotton leaves, respectively. Increased production of heliocides (C_{25} terpenoid aldehydes) were found in the volatile recepient wounded or undamaged cotton leaves. The heliocides are natural insecticides presumed localized in the subepidermal pigment glands in leaves. Myrcene, a volatile precursor of heliocide H_2, also caused significant increases in heliocide production when leaves were exposed to the volatilized chemical.

INTRODUCTION

Baldwin and Schultz [1] damaged leaves of potted poplar (*Populus* × *euroamericana*) ramets and sugar maple (*Acer saccharum*) seedlings and demonstrated increased concentrations of phenolic compounds in the damaged leaves and also in the leaves of undamaged plants sharing the same enclosure. Recently, Rhoades [2] found that Sitka willow (*Salix sitchensis*) trees attacked by tent caterpillars (*Malacosoma californicum·pluviale*) and nearby unattacked control trees exhibited altered leaf quality. Fall webworms (*Hyphantria cunea*) fed the attacked leaves grew more slowly than those fed leaves from unattacked willows. Both references suggest that an airborne signal originating in damaged tree tissues may stimulate biochemical changes in neighbouring undamaged trees that could influence the feeding and the growth of phytophagous insects.

Bell [3] induced accumulation of phloroglucinol-reactive compounds in tissues of cotton (*Gossypium hirsutum* or *G. barbadense*) by inoculating with conidia of *Verticillium albo-atrum* or with sporangiospores of *Rhizopus nigricans*. He described the accumulation of these phloroglucinol-reactive phenolic compounds in terms of 'gossypol equivalents' and discussed their accumulation relative to that of phytoalexin production in other microbial–host plant interaction systems.

Sesquiterpenoids structurally related to gossypol occur in the subepidermal pigment glands of *Gossypium hirsutum* and are toxic to *Heliothis* spp. These have been indentified as hemigossypolone, the predominant terpenoid in young leaves of *G. hirsutum*, and as the derived terpenoids, heliocides H_1, H_2, H_3 and H_4, which replace hemigossypolone as the leaf matures [4].

The purpose of this current investigation was to determine if bioactive volatiles coming from damaged cotton leaves, fungal cultures or from fungal-infected cotton leaves can alter leaf chemistry in leaves sharing the same enclosure and to identify those chemical changes and those volatiles inducing changes in leaf chemistry.

RESULTS AND DISCUSSION

After a 1 week test period, volatile transmitting leaves, volatile receiving leaves and control leaves were collected from separate desiccators and the 'gossypol equivalents' were determined on the lyophilized leaf extracts. 'Gossypol equivalents' determinations were used as a measurement of the quantitative increase of phenolic compounds induced under the described treatment procedures. Wounded leaves exposed to volatiles from wounded leaves inoculated with *A. flavus* showed a 52% significant average increase of 'gossypol equivalents' over that of wounded control leaves. Also non-wounded leaves exposed to volatiles from wounded leaves inoculated with *A. flavus* showed a 34% significant average increase of 'gossypol equivalents' over that of control non-wounded leaves (Table 1). Leaves exposed to volatiles from *A. flavus* liquid cultures or from volatiles from cut leaves did not show significant increases at ($P = 0.05$) when compared to control leaves.

Silica gel TLC plates spotted with extracts from leaves exposed to volatiles of *A. flavus*-infected leaves, developed in solvent 1 (see Experimental) and sprayed with the phloroglucinol reagent showed seven phloroglucinol-reactive spots. The R_fs and colours formed were: 0.11 (violet), 0.16 (maroon), 0.27 (magenta), 0.33 (orange), 0.39 (orange), 0.43 (pink) and 0.74 (yellow-green). Extracts from leaves which were the recipients of the volatiles all contained elevated levels of the orange coloured spots, compared to extracts from control leaves. The magenta coloured spot (hemigossypolone) and the orange coloured spots (heliocides H_1, H_2 and H_3) were identified by comparing TLC R_f values, colour derivatives formed with acidic phloroglucinol, by maximum UV-visible absorptions with those of purified components, and by mass spectral measurements [4–6]. TLC comparisons showed that heliocide H_2 was the predominant compound that accumulated in leaves exposed to volatiles from *A. flavus*-inoculated leaves. Spots at lower R_fs on TLC plates also contained unidentified phloroglucinol-reactive com-

Table 1. Effects of volatile chemicals emitted by infected or wounded cotton leaves or by *A. flavus* cultures on the 'gossypol equivalent' content of receptor cotton leaves after 7-days exposure

		Treatment condition means* in mμ mol 'gossypol equivalents'/g dry leaf tissue		
Volatile source	Volatile receptor	Source leaves	Receptor leaves	Control leaves†
Cut leaves	Normal leaves	337 a‡	225 b	197 b
A. flavus inoculated leaves	Wounded leaves	1003 c	702 c	337 d
A. flavus inoculated leaves	Normal leaves	481 e	339 f	225 g
A. flavus cultures	Normal leaves		295 h	229 h
A. flavus cultures	Wounded leaves		401 i	317 i

*There were at least three replicates of each treatment condition, and the data represent the means of the analyses of three subsamples in each treatment.

†Control leaves treated the same as receptor leaves, except they received only filtered compressed air.

‡Means in separate columns in each treatment followed by the same letter are not significantly differed at $P = 0.05$ according to Ducan's Multiple Range Test.

pounds but these compounds did not increase in the volatile-recipient leaves.

When authentic myrcene was used as the volatile source, an 18–20-fold increase in heliocide H_2 was noted in treated leaves compared to those that received only purified air. Myrcene is a major volatile terpene (over 8% of the total terpene) in the essential oil of glanded flower buds of cotton [7]. In our laboratory, we have found that myrcene represents 17.7% of the total volatiles in non-wounded Acala SJ-2 leaves and 24.8% of the total volatiles in wounded Acala SJ-2 leaves. Heliocide H_2 is formed naturally by a Diels–Alder addition of hemigossypolone and myrcene in the pigment glands of the cotton plant [6]. Myrcene reacts with hemigossypolone *in vitro* at room temperature giving predominately heliocide H_2 [6]. My observation shows that volatile myrcene also induces the production of heliocide H_2 as the predominant product in exposed leaves. Leaf damage caused by *A. flavus* penetration might release myrcene from the pigment glands, and by reacting with hemigossypolone, myrcene could increase the accumulation of heliocide H_2 in volatile-recipient leaves. The increased production of heliocide H_2 alone cannot quantitatively explain the increased production of phloroglucinol-reactive compounds which occurred in volatile recipient leaves. Utilizing the detection techniques in this experiment, only heliocide H_2 accumulations were apparent. Further separation techniques are in progress to isolate and identify any other phloroglucinol-reactive compounds which may accumulate.

EXPERIMENTAL

Fungi and culture conditions. Aspergillus flavus (SRRC 1000) were maintained on potato-dextrose agar plates. *A. flavus* spores (2.4×10^3 in 10 μl distilled water) was placed on wounded areas of surface-sterilized cotton leaves. Adye and Mateles [8] liquid medium was inoculated with *A. flavus* spores for production of fungal volatiles. All cultures and experiments were maintained or performed at 26 ± 2°.

Plants and pre-test treatment of detached leaves. Young third or fourth true leaves with their petioles were detached from 2-month-old post-emergence Acala SJ-2 cotton plants (*Gossypium hirsutum*) grown under greenhouse conditions. Detached leaves and attached petioles were surface-sterilized by successive immersions for 30 sec in 20% Clorox, 70% EtOH and sterile distilled H_2O. Wounds in leaves were produced by slight abrasion on three separate areas on the upper surface of each leaf; each wounded area was approximately 6 mm in diameter.

Apparatus. In each test 20 surfaced-sterilized cotton leaves were placed in each of four sterilized 150 mm ID desiccators equipped with desiccator covers containing sleeve valves (Fig. 1). Two C-clamps were positioned over each desiccator's cover flange and body flange in order to hold the positive air pressure of the tests. The petioles of each leaf were placed through the holes of the porcelain desiccator plate, and the basal areas immersed in sterile Hoagland's plant nutrient solution [9]. The volatile-transmitting desiccator (A) contained either wounded leaves, wounded leaves treated with fungal spores or fungal cultures. Compressed air at a head pressure of 5 psi was microbial filtered through a Pall Ultiporμ (0.2 μm) sterile gas filter, and the filtered air was directed into the desiccator by a 19 gauge stainless steel 10-cm-long syringe needle. A plastic tube was added to the needle to extend the air flow to 2.5 cm above the porcelain desiccator plate. The needle was introduced into a plastic tube which linked desiccator (A) with the volatile-receiving desiccator (B), and the needle was positioned to enter desiccator (A) at the sleeve valve. Air from the desiccator (A) passed through the sleeve valve and was filtered through a second Pall Ultiporμ (0.2 μm) sterile gas filter in line to desiccator. Exhausted air from desiccator (B) was received by a similar 19 gauge syringe needle fitted with the same length of plastic tubing and introduced into the receiving container in the same manner as described for desiccator (A). Exhausted air was adjusted to a flow rate of 30 cm^3/min and was exhausted under 3 cm of water. Wounded and non-wounded leaf controls were tested in identical apparati using separate microbial-filtered compressed air supplies.

The desiccators received 12 hr of illumination per day from a bank of eight 15-W fluorescent Plant Gro lights positioned 20 cm above the containers. All experimental tests lasted 7 days.

Determination of 'gossypol equivalents' in leaves. Petioles were cut from leaves and leaves were rinsed with cold distilled water, placed in a freezer at −60° for 1 hr, lyophilized and weighed. 200 ml of 50% aq. EtOH was added to 20 lyophilized leaves per treatment, and this mixture was blended in a homogenizer for 3 min. Filtered extracts were diluted to 40% EtOH and were

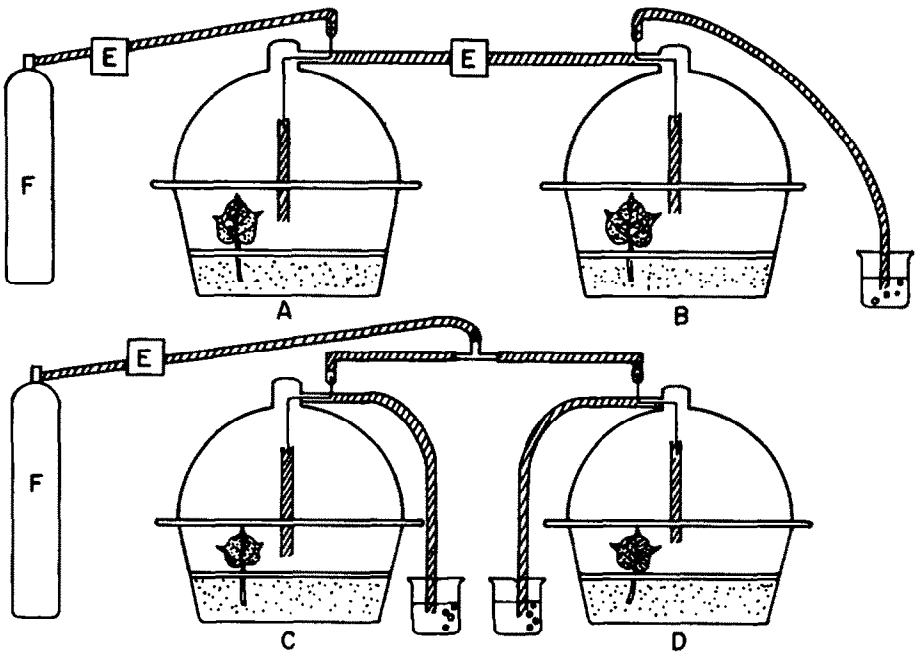

Fig. 1. Diagram of apparatus. A, Volatile emitting container; B, volatile receiving container; C, non-wounded control container; D, wounded control container; E, microbial traps; F, compressed air cylinders.

extracted 3 × with one, one-half and one-half volumes of ethyl ether in a separatory funnel. Ether extracts were washed 3 × with equal volumes of distilled water and reduced to dryness under N_2 purging and vacuum. Gossypol equivalent concentrations were determined by the Storherr and Holley technique [10] as modified by Bell [3]. The dry residue remaining after removal of ether was dissolved in aq. 50% EtOH and 1 ml of this EtOH soln was mixed with 0.5 ml of 5% phloroglucinol in EtOH and 1 ml conc HCl. After 30 min, the mixture was diluted to 10 ml and the absorbance was determined at 550 μm. 'Gossypol equivalents' were determined by comparison with a standard curve determined with authentic gossypol. All leaf extractions and spectrophotometric determinations were performed in subdued illumination.

Identification of phloroglucinol-reactive compounds. Leaves were extracted by a modification of the method described by Bell and Stipanovic [5]. Equal weights of fresh leaves from each treatment were placed in 500 ml flasks, and the leaves submerged in 150 ml of extracting solvent (40% EtOAc: 60% ethanolic bisulphite solution). The flasks then were placed on a reciprocating shaker for 12 hr. 270 ml of a soln containing 9% NaCl and 1% conc HCl was added to each flask, and the organic layer formed was removed from the top of each flask. Equal volumetric amounts of each organic layer were spotted on silica gel TLC plates and developed in hexane–EtOAc–HOAc (90:10:0.5), solvent 1, or in hexane–Et_2O–HCO_2H (85:15:1), solvent 2. After drying for 5 min, the developed plates were sprayed with phloroglucinol reagent (equal volumes 5% phloroglucinol in EtOH and conc HCl) to determine the qualitative distribution of the phloroglucinol-reactive compounds.

To identify heliocides H_1, H_2, and H_3, prep. silica gel TLC plates were streaked with leaf extracts and developed in solvent 2. Areas between R_fs 0.3 and 0.5 were scraped from the plates and the compounds were eluted from the silica gel with EtOAc. The product was further purified by prep. TLC with several developments in solvent 1 [4]. The yellow band at R_f 0.25 was a mixture of heliocides H_2 and H_3, the light yellow band at R_f 0.35 was heliocide H_1. Heliocide H_2 was further purified from hexane and the UV spectrum scan and mass spectra measurements of the preparation agreed with a simular published scan for heliocide H_2 [6].

Assaying the monoterpene myrcene as a bioactive volatile. Microbial-filtered compressed air was circulated through a desiccator containing 2 ml of myrcene in a 9-cm wide Petri dish, and the resulting volatiles were exhausted to a desiccator containing 20 young cotton leaves. Leaves under compressed air only served as a control. After 1 week, equal weights of treated and control leaves were collected and concentrations of terpenoid aldehydes were determined. The quantitative estimate of heliocide H_2 was determined spectrophotometrically on purified eluents from TLC prep. plates using published molar extinction coefficients (ε) [6].

Statistical evaluations. There were at least three replicates of each treatment condition, and the data (Table 1) represent the means of the analyses of three subsamples in each treatment. Significant differences were determined with Ducan's Multiple Range Test [11] which tests all pairs of means.

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