Quantification of Volatile Terpenes of Glanded and Glandless *Gossypium hirsutum* L. Cultivars and Lines by Gas Chromatography

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Volatile terpene content of *Gossypium hirsutum* L. glanded and glandless cultivars and lines was determined by solvent extraction and analysis by capillary GC. Identity of the various peaks was confirmed with GC–MS by comparing the mass spectra of unknowns with those of authentic samples. Data showed that glandless samples lacked or contained small quantities of volatile terpenes as compared to the glanded. The importance of these volatiles in attracting herbivores and parasites is considered.

In the late 1960s and early 1970s researchers characterized the volatile chemical composition of *Gossypium hirsutum* L. var. Deltapine Smoothleaf. Minyard et al. (1965, 1966) identified the monoterpenes α-pinene, L-camphene, β-pinene, myrcene, α-phellandrene, α-terpinene, L-limonene, β-phellandrene, *trans*-β-ocimene, γ-terpinene, and terpinolene and the sesquiterpenes copaene, L-*trans*-α-bergamotene, L-caryophyllene, farnesene, α-humulene, *cis*-γ-bisabolene, δ-guaiene, and δ-cadinene. Minyard et al. (1968) also identified a new sesquiterpene alcohol, β-bisabolol, in Deltapine cotton. Subsequently, Thompson et al. (1971) noted that Deltapine cotton also contained the sesquiterpene β-caryophyllene oxide.

Hedin et al. (1972) found that *Gossypium barbadense* var. Giza 69 (Egyptian cotton) contained all of the sesquiterpenes found in Deltapine cotton except β-bisabolol. They also reported that the sesquiterpene content was much higher in Giza cotton than in Deltapine cotton. In addition, eight minor monoterpenes found in Deltapine cotton were not found in Giza cotton.

Elzen et al. (1983, 1984) isolated and identified sesquiterpenes in the essential oil of *G. hirsutum* var. Stoneville 213, during a study demonstrating the attraction of a parasitic wasp to a host's food source. Stoneville 213 cotton contained the attractive terpenes α-humulene, γ-bisabolene, β-caryophyllene oxide, β-bisabolol, gossonorol (a new compound), and spathulenol (a compound isolated for the first time from cotton). β-Bisabolol was the major alcohol present. β-Caryophyllene was isolated but was not attractive to the wasps. We recently (unpublished results) identified the monoterpenes α-pinene, myrcene, and ocimene in the essential oil of Stoneville 213 cotton.

Genetic cultivars and lines of cotton lacking lysigenous pigment glands (i.e., glandless) have been developed by plant breeders. In most glandless lines, the mutant alleles gl_2 and gl_3 have been introduced in place of the dominant Gl_2 and Gl_3 alleles to give a plant completely devoid of glands (Bell and Stipanovic, 1977). Removing pigment glands generally increases plant damage by insects (Jenkins et al., 1966, Lukefahr et al., 1966). In cotton bracts and leaves, various toxic terpenoid aldehydes, including gossypol, heliocide H_1, and heliocide H_2, occur only in lysigenous pigment glands and thus do not occur in glandless cotton cultivars and lines. Certain volatile terpenoids are intermediates in terpenoid aldehyde synthesis (Bell and Stipanovic, 1977) and thus probably also occur in pigment glands, but their occurrence in specific tissues is uncertain.

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The present study is part of the continued effort to identify the volatile components of the essential oil of various *Gossypium* varieties and cultivars and to identify the role they may play in influencing the behavior of parasitoids and herbivores. We compared the volatile terpene profiles of several glanded and glandless cotton pairs, because the glands are known to produce terpenoid aldehydes and quinones that are absent from glandless cotton plants.

METHODS AND MATERIALS

Fresh cotton terminal leaves (ca. 6 g) were weighed to the nearest 0.1 g, and ethyl ether (pesticide analysis grade) was added to produce a final concentration of 50 mg/mL. Samples were held in covered 250-mL beakers under a fume hood for 1 h, and then the solution was decanted into 250-mL ground-glass stoppered flasks. The remaining cotton leaves were washed briefly with 50 mL of ethyl ether that was decanted and combined with the original extract. Extracts were then vacuum evaporated in a 20 °C water bath to near dryness. One milliliter of ethyl acetate/hexane (10:90) was added to redissolve the residue, and the solution was added to a dry, 2-cm column of inactivated Florisil in a disposable Pasteur pipet. The samples were eluted with 4 mL of developing solvent (EtOAc/hexane (10:90)). This procedure yielded yellow, light green, or clear samples and left various brown, black, dark green, and bright yellow residues on the column.

Individual samples (0.4 μL) prepared by the above procedures were applied to a 0.3 mm × 25 m vitreous silica BPl capillary column in an FID-equipped Varian 3700 gas chromatograph with SGE on-column injection. Nitrogen pressure was 10 psig, under a temperature program of 60 °C (4-min delay) to 180 °C final temperature (7 min at final) at 10 °C/min. Mass spectral data were obtained with a Finnigan guadrupole 1020 GC–MS, with a 0.3 mm × 25 m vitreous silica BPl column and on-column injection. Helium pressure was 10 psig, and program conditions were identical with those previously stated. The identity of the various peaks was confirmed by comparing the mass spectra of unknowns with those of authentic samples obtained as gifts, synthesized, or purchased. GC peaks integrated in each sample were corrected for detector response by applying known quantities of standard terpenes. Identified terpenes in individual samples were quantified by calculation of GC response to known terpene standards and comparison to sample responses.

At a later date, we developed methods for sampling and analyzing cotton glandular contents. Gland contents of ACALA SJ-1 flower bud calyxes were obtained by puncturing the gland surface with a fine-pointed capillary tube. An oily golden-colored material entered the tube by capillary action when glands were punctured. Capillaries

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Table I. Relative Percentages[a] of Compounds (ppm[b] in Parentheses) in Leaves of Five Pairs of Glanded (Gl) and Glandless (gl) Cultivars and Lines of Cotton

compd	ret time, min	CAMD-E (Gl)	GN-8 (gl)	PAY-202 (Gl)	PAY-784 (gl)	Sto-213 (Gl)	Sto-209 (gl)	LANK-57 (Gl)	GL-6 (gl)	ACALA SJ-1 (Gl)	ACALA 8160 (gl)
unknown	6.10	0	0	0	0	0.7	0	0.6	0	0.3	0
α-pinene	6.85	8.6 (94)	0	11.8 (557)	100 (9)	13.5 (152)	8.7 (1)	12.6 (669)	5.9 (1)	13.2 (526)	0
β-pinene	7.77	2.1 (23)	0	3.0 (145)	0	3.0 (34)	0	3.2 (171)	0	3.0 (121)	0
myrcene	8.05	9.1 (100)	0	9.8 (461)	0	8.3 (93)	34.1 (3)	12.4 (655)	4.9 (1)	11.2 (447)	0
limonene	8.89	0.8 (9)	0	1.1 (50)	0	1.2 (13)	0	1.4 (75)	0	1.2 (49)	0
ocimene	9.25	5.0 (55)	0	5.2 (246)	0	5.1 (58)	0	5.0 (265)	0	7.6 (304)	0
α-copaene	15.17	1.5 (17)	0	0.9 (45)	0	1.5 (17)	0	1.7 (89)	0	1.6 (63)	72.8 (2)
β-caryophyllene	15.82	33.6 (370)	0	27.5 (1309)	0	26.7 (303)	35.0 (3)	26.4 (1408)	28.1 (10)	26.7 (1075)	27.7 (1)
unknown	15.96	0.4	0	0.5	0	1.7	0	0.7	0	0.5	0
unknown	16.12	0.4	0	0.4	0	0.9	0	0.4	0	0.3	0
α-humulene	16.29	11.5 (127)	0	8.7 (414)	0	9.2 (104)	0	8.8 (468)	21.1 (7)	8.5 (342)	0
unknown	16.66	1.3	0	1.3	0	1.4	0	1.5	0	1.5	0
unknown	16.89	3.2	0	5.3	0	0.3	0	0.6	0	0.7	0
γ-bisabolene	17.10	6.3 (70)	0	8.9 (432)	0	8.4 (98)	10.8 (1)	8.4 (460)	16.9 (6)	8.2 (335)	0
unknown	17.22	0.7	0	0.6	0	1.2	0	0	0	1.1	0
spathulenol	17.73	0.2 (3)	0	0.4 (17)	0	0.2 (3)	0	0.4 (20)	0	0.3 (13)	0
unknown	18.14	0.6	0	0	0	0	0	0	0	0	0
β-caryophyllene oxide	18.29	0.7 (7)	0	0.6 (26)	0	0.2 (2)	0	0.3 (16)	23.1 (8)	0.3 (10)	0
gossonorol	19.08	0.3 (3)	0	0	0	1.2 (13)	0	0.3 (15)	0	0.2 (8)	0
β-bisabolol	19.97	13.8 (151)	0	14.2 (697)	0	15.4 (175)	11.4 (1)	15.4 (822)	0	13.8 (554)	0
rel total,[c] %		20.6	0	89.2	0.2	21.2	0.2	100	0.7	75.4	0.1
total ppm terpenes		(1029)	0	(4399)	(9)	(1065)	(9)	(5133)	(33)	(3847)	(3)

[a] Determined by dividing the area of the individual peak ×100 by the total area of all peaks obtained between retention times 6–20 min for the variety or line in question. [b] Determined by quantification with known standard terpenes, and adjustment for ppm in total volume of sample extract. [c] Determined by giving a value of 100 to the total area of all peaks for LANK-57 (sample with the largest real area) and calculating the total area of all peaks of other samples as a percentage of the LANK-57 total.

Table II. Volatile Terpenes Identified in ACALA SJ-1 Gland Contents

compd	ret time, min	amt, ppm
α-pinene	6.85	11580
β-pinene	7.77	1929
myrcene	8.05	2572
limonene	8.89	643
ocimene	9.25	252
α-copaene	15.17	955
β-caryophyllene	15.82	3215
α-humulene	16.29	1286
γ-bisabolene	17.10	643
spathulenol	17.73	386
β-caryophyllene oxide	18.29	129
gossonorol	19.08	386
β-bisabolol	19.97	7074

were prepared by drawing melting point tubing in a Brinkman automatic capillary puller. A total of 70 glands were sampled in this manner. The volume sampled was estimated by drawing an authentic sample of pure β-caryophyllene to the level of the previously collected gland sample in the capillary, expelling the β-caryophyllene into 20 μL of hexane, and analyzing a 0.5-μL aliquot by capillary GC. The integrated area obtained for this aliquot was compared to the area obtained for a standard 1 μg/μL solution of β-caryophyllene, and the quantity of unknown was calculated. This quantity was corrected for dilution and multiplied by the specific gravity (density) of β-caryophyllene. Calculations indicated that about 2.5 μL of sample was obtained from 70 glands. The gland sample was analyzed by capillary GC under identical conditions noted previously. A similar volume of fluid was obtained by puncturing adjacent calyx tissue and bract veins (avoiding all glands) and analyzing the sample by capillary GC to determine the presence of volatile terpenes.

Commercial standards were purchased from Givaudan Corp. (β-caryophyllene oxide), CA Aromatics Co. (β-caryophyllene, γ-bisabolene), and Fluka A. G. (humulene) or were obtained as gifts (spathulenol, copaene). Gossonorol was synthesized by the procedure shown in Elzen et al. (1984). Monoterpene standards (pinene, ocimene, limonene, myrcene) were purchased from Aldrich Chemical Co.

Under program conditions stated above, the following retention times (in minutes) were obtained for hydrocarbon reference standards C_9–C_{18}, respectively: 5.95, 8.32, 10.38, 12.20, 13.84, 15.32, 16.85, 18.47, 20.29, 24.39.

The following *G. hirsutum* cultivars and lines (glanded, glandless) were grown in small field plots: Tamcot CAMD-E, Tamcot GN-8; ACALA SJ-1, ACALA 8160; Paymaster 202, Paymaster 784; Lankart 57, Rodgers GL-6; Stoneville 213, Stoneville 209.

RESULTS AND DISCUSSION

The major volatile terpenes identified in glanded cotton varieties (*G. hirsutum*) and their relative percentages (based on total peaks integrated) are shown in Table I. In general, the percentages of these compounds in CAMD-E, Stoneville 213, Paymaster 202, Lankart 57, and ACALA SJ-1 were similar. Direct comparison of individual compound percentages in varieties in the present study with the previously published data on Giza 69 and Deltapine Smoothleaf varieties (Hedin et al., 1972); Minyard et al., 1965, 1966; Thompson et al., 1971) is not possible due to differences in extraction procedures and total number of components reported. However, with the exception of gossonorol and spathulenol, which were isolated for the first time from cotton by our group (Elzen et al., 1984), all components present in samples in our study were previously reported in Deltapine Smoothleaf cotton (Minyard et al., 1965, 1966; Thompson et al., 1971). Myrcene was absent from *G. barbadense* var. Giza 69 (Hedin et al., 1972).

Table I gives the data for terpene content in glanded cotton and the most nearly comparable glandless pair. Glandless lines either lacked terpenes and unidentified compounds or had only small amounts. Relative volatile chemical content was below 1% in all glandless lines. Also, glandless samples contained an average of 11 ppm of volatiles, as compared to an average of 3094 ppm in glanded samples. These data strongly indicate that the volatile terpenes are produced and stored in the glands of *Gossypium* species. The glanded lines all had similar amounts of identified terpenes with the exception of a lack of gossonorol in Paymaster 202.

The volatile terpenes found in cotton leaves were present in gland contents of ACALA SJ-1 (Table II). No volatile terpenes were found in tissues adjacent to these glands by identical chromatographic procedures. These data strongly indicate that the volatile terpenes are produced and stored in the lysigenous glands.

The finding that volatile terpenes are apparently part of the contents of pigment glands may have important implications. The response of the boll weevil to cotton terpenes was characterized previously (Hedin et al., 1973). However, the potential allelochemical, repellent, or attractive properties of the volatile mono- and sesquiterpenes to most insects and other herbivores are unclear. There is an interest in manipulating the terpenoid aldehydes (gossypol, heliocides, raimondal) for resistance in cotton to *Heliothis* species. Breeding for increased allelochemicals could also be coupled to alteration of volatile terpenes in cotton, thereby resulting in increased host plant resistance through nonpreference.

Both terpenoid aldehydes and certain volatile terpenes have been shown to cause inflammatory responses in mast and macrophage cells of animals (Elissalde et al., 1983). Thus, these compounds might be involved in the etiology of byssinosis, a respiratory disease of cotton mill workers. Comparisons of animal and human responses to dust from glandless and glanded cotton lines should help evaluate the importance of terpenoids in the disease, since these compounds are absent from the glandless lines.

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Registry No. α-Pinene, 80-56-8; β-pinene, 127-91-3; myrcene, 123-35-3; limonene, 138-86-3; ocimene, 7216-56-0; α-copaene, 3856-25-5; β-caryophyllene, 87-44-5; α-humulene, 6753-98-6; γ-bisabolene, 495-62-5; spathulenol, 6750-60-3; β-caryophyllene oxide, 1139-30-6; gossonorol, 92691-77-5; β-bisabolol, 15352-77-9.

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Nitrosation of Phenol and 2,6-Dimethoxyphenol and Its Effect on Nitrosamine Formation

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We measured modifying effects of the wood smoke constituents phenol and 2,6-dimethoxyphenol (syringol) on the rates of *N*-nitrosomorpholine (NMOR) and *N*-nitrosopyrrolidine (NPYR) formation in vitro. The second-order nitrosation rate constants (pH 3.0, 37 °C) were 127 M^{-1} min^{-1} for syringol and 1.16 M^{-1} min^{-1} for phenol. Under our standard conditions, syringol and ascorbic acid blocked NMOR formation by 91 and 77% and blocked NPYR formation by 71 and 79%, respectively. Phenol did not affect NMOR formation but enhanced NPYR formation by 358%. These results indicate that basicity of the amine and hence the rate of nitrosamine formation may alter the rate-modifying effect of phenol considerably. The results show that syringol can be a potent nitrosation inhibitor.

Formation of carcinogenic nitrosamines is influenced by various factors, including the presence and nature of modifying agents (Issenberg and Virk, 1974; Mirvish, 1975; Douglass et al., 1978; National Academy of Sciences, 1981). Phenols are important modifiers of amine nitrosation, because they are widely distributed in the environment. Furthermore, they are usually reactive with the nitrosating species, with which they react irreversibly to form *C*-nitroso products that may catalyze *N*-nitrosamine formation under acidic conditions (Davies and McWeeny, 1977; National Academy of Science, 1981; Walker et al., 1982). Reactive phenols compete with amines for the available nitrosating agents (Nakamura and Kawabata, 1981; Pignatelli et al., 1984; Kuenzig et al., 1984) and have been found either to enhance or to block *N*-nitrosoproline (NPRO) formation under different conditions (Pignatelli et al., 1982).

Nitrosation of phenol occurs readily in acidic solutions of sodium nitrite, yielding mainly *p*-nitrosophenol, with about 8–10% of the ortho compound (de la Mare and Ridd, 1959; Morrison and Turney, 1960). Substitution of electron-releasing groups such as alkoxyls, particularly at the 2- and/or 6-positions of the phenolic ring, appears to stabilize the positive charge on the carbon atom para to the hydroxyl group and may enhance the nitrosation rate of the phenolic compound.

Issenberg and Virk (1974) reported the blocking of *N*-nitrosomorpholine (NMOR) formation from morpholine (MOR) and nitrite by phenol and syringol, two phenolic components of wood smoke and smoked foods (Lustre and Issenberg, 1970). The nitrosation kinetics of phenol has been studied previously under different conditions (Suzawa et al., 1955; Morrison and Turney, 1960; Challis and Lawson, 1971; Challis, 1973), but the nitrosation kinetics of syringol has not been investigated.

The kinetics of nitrosation of phenol and syringol was examined in the present study to assess the possible role of the phenols in modifying nitrosamine formation rates. The nitrosation of MOR, a weakly basic and easily nitrosatable secondary amine, and of pyrrolidine (PYR), a strongly basic and slowly nitrosatable amine (Mirvish, 1975), was examined. Ascorbic acid was included as a reference inhibitor.

MATERIALS AND METHODS

Reagents. MOR, PYR, and syringol were purchased from Aldrich Chemical Co., Milwaukee, WI; and phenol was purchased from Mallinckrodt, St. Louis, MI; sodium nitrite, from Baker Chemical Co., Phillipsburg, NJ; and ascorbic acid from Fisher Scientific Co., Fair Lawn, NJ. Dichloromethane (DCM) was "distilled in glass" from Burdick and Jackson Inc., Muskegon, MI. NPYR solution (100 g/mL in 2,2,4-trimethylpentane) was purchased from Thermo Electron Co., Waltham, MA; and NMOR was provided by Dr. S. Mirvish (Eppley Institute). All other chemicals were reagent grade materials from commercial sources.

Nitrosation of Phenols. The reaction mixture (147 mL), containing phenol or syringol (4×10^{-4} M), and sodium nitrite (4×10^{-4} M), was adjusted to pH 3.0 with perchloric acid (approximately 6.8×10^{-4} M) and maintained at 37 °C in a water bath. Twenty-milliliter portions, drawn at intervals of 0, 20, 44, 68, 92, and 164 h for phenol and 0, 5, 10, 15, 20, 25, and 50 min for syringol, were transferred to a mixture of 0.5 mL of 1 M Na_2SO_3 (Mirvish et al., 1972) and 0.48 mL of 1 M $HClO_4$ to stop the reaction. A separate experiment, described below, evaluated the effectiveness of sulfite in removing nitrite. Unchanged

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