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Constituents of Cotton Seedlings; an Investigation of the Preference of Male Boll Weevils for the Epicotyl Tips

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The essential oil obtained from the epicotyls (apical tips, 5–8-mm section from apex) of cotton seedlings (*Gossypium hirsutum* L. var. Deltapine Smoothleaf) was analyzed with an integrated gas chromatography–mass spectrometry system. Structures for 36 compounds are proposed: six sesquiterpene hydrocarbons, seven esters, four alcohols, 14 oxygen-containing compounds, and five miscellaneous. Four of the five miscellaneous compounds are believed to be metabolic products of the fungicide Terraclor with which the cotton seed had been treated. Partial structural data for some additional compounds are also given. A number of the compounds are present in cotton bud essential oil, but there are significant differences. The degree of preference of boll weevils, *Anthonomus grandis* Boheman, for cotton seedlings and their constituent parts was evaluated. Primarily males were attracted to the epicotyls and hypocotyls, but they fed exclusively on the epicotyls and were strongly deterred from feeding on the hypocotyls. Although the epicotyls appear to possess some unique quality that encourages feeding and pheromone production, none of the identified components seemed adequate to explain these properties.

Maxwell *et al.* (1963) reported that the flowers and buds were the most favored parts of the cotton plant for feeding by boll weevils (*Anthonomus grandis* Boheman), but they also fed well on whole seedlings. Later, a definite feeding preference was shown for the epicotyl of the seedling over that for the cotyledon, hypocotyl, and radical (Jenkins and Parrott, 1972). Then, since male boll weevils fed fresh cotton or cotton seedlings are more attractive than those fed artificial diets, this tissue appeared to be suitable for investigation because it might contain a specific precursor of the boll weevil sex attractant complex identified by Tumlinson *et al.* (1969); also, it might not contain as large a spectrum of compounds as the mature plant since it is a tissue which appears at an early stage in the development of the plant.

The present investigation was therefore made to identify as many of the volatile components in the epicotyl essential oil as possible so that they might be compared with the components of the sex attractant (Tumlinson *et al.*, 1969) and those of the essential oil of the cotton bud (Hedin *et al.*, 1971a,b, 1972; Minyard *et al.*, 1965, 1966, 1967, 1968). Feeding stimulant bioassays were performed to confirm the previous unreported data, this time with both sexes separately. Plant attractant bioassays were also performed with both sexes separately to further define the insect feeding preference.

MATERIALS AND METHODS

Plant Material. Treated delinted commercial cotton seeds were germinated in the greenhouse in flats of vermiculite and harvested 6 days after planting (total of 5 kg). When the cotyledons had been detached, the epicotyl sections of the seedlings were removed and immediately immersed in absolute methanol to prevent further enzymatic activity. They were then stored at 0° until an adequate quantity was obtained for steam distillation.

Feeding Stimulant Bioassays. Assays of the components as feeding stimulants were conducted as described by Hedin *et al.* (1968). Aqueous solutions (0.5 g/ml of concentration) of total seedlings, cotyledons, epicotyls, hypocotyls, and radicles were assayed. Ten 5-day-old laboratory-reared and sexed boll weevils (sexes were tested separately) were placed in a 100-mm petri dish with two agar plugs, one wrapped with paper impregnated with the test compound and the other with paper that was not impregnated. The dishes were placed in an incubator that was maintained at 28° and equipped with balanced lighting, and the weevils were allowed to feed for 4 hr. When the papers were unwrapped from the plugs, any punctures

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Table I. Feeding Stimulant Assay with Boll Weevils on Cotton Seedlings and Dissected Plant Organs

Plant organ	Mixed weevils, *T/S*	Female weevils, *T/S*	Male weevils, *T/S*
Whole seedlings	+3	−13	+58
Cotyledon	+23	−16	−100
Epicotyl	+24	+21	+90
Hypocotyl	+11	+12	−98
Radicle	−35	−79	+43

Table II. Plant Attractant Bioassay with Boll Weevils on Cotton Seedlings and Dissected Plant Organs

Sample	% males responding to		% females responding to	
	Test	Blank	Test	Blank
Whole seedling	35.0	1.3	28.8	3.8
Cotyledon	21.3	1.3	5.0	8.8
Epicotyl	46.3	1.3	18.8	1.3
Hypocotyl	40.0	5.0	27.5	3.8
Radical	13.8	3.8	7.5	2.5
Grandlure[a]			50.0	1.3

[a] Mixture of four components, 8 μg total; Tumlinson *et al.* (1969).

visible on the underside were counted. Each test was replicated three times. The mean number of punctures in the blank (not impregnated) was subtracted from the mean number in the test plugs to produce a positive or negative score; this score was then converted to an index (*T/S*) by multiplying by 100 and dividing by the response of similarly exposed weevils to an aqueous extract of fresh cotton buds.

Plant Attractant Bioassay. All bioassays of the components as plant attractants were performed as previously described (Hardee *et al.*, 1966, 1967). Quantities of each plant part were weighed and ground in a blender with an equal weight of water. Then the contents were brought to a boil to inactivate enzymes and filtered, and the filtrate was frozen. For bioassay, 1 ml was pipetted onto firebrick and 20 sexed insects were placed in the bioassay device for 1 hr. Insects responding to the test firebrick and the blank were then counted. Each test was replicated four times. For comparison, a test was made in which females were similarly exposed to 8 μg of grandlure.

Isolation and Fractionation of Epicotyl Essential Oil. Epicotyls (767 g) were steam-distilled in an all glass system, and the distillate was extracted with ethyl ether. Yield of oil was *ca.* 0.30 g (399 ppm). The oil was chromatographed on a 2 × 25-cm cold water-jacketed Florisil column. The hydrocarbons were eluted with 100 ml of pentane, and the polar compounds were eluted successively with 100 ml each of 5, 10, 20, and 50% Et_2O in pentane and finally with 100% Et_2O. Progress of the elution and recombination of all fractions into reconstructed fractions was monitored by silica gel tlc.

Analytical Glc–Ms. Fractions were introduced into a Hewlett-Packard 5930 quadrapole mass spectrometer from a 250 ft × 0.03 in. capillary column coated with OV-17. The glc unit was programmed from 120 to 160° at 1°/min; the final temperature was maintained for 60 min. Mass spectra were obtained at 70 eV. The gas chromatographic profile obtained with an FID was used to estimate the relative concentrations of the oil components. Material balance observations were made by peak triangulation and normalization to 100%.

RESULTS AND DISCUSSION

The data for the bioassay of feeding stimulants (Table I) support the results reported by Jenkins and Parrott (1972); the insects were primarily attracted to the epicotyl. However, our test with single sexes showed that the males primarily are stimulated to feed on this tissue. The bioassay of plant attraction (Table II) produced similar results: the attraction of males to the epicotyl was twice as great as that of the females. The hypocotyl was almost as attractive as the epicotyls, but the males were nevertheless strongly repelled from feeding on it. Thus, the epicotyls seem to possess some unique properties that first attract the insect and then initiate feeding. Indeed, the 46% response of males to the epicotyl can be considered a maximum response since grandlure, in this test, evoked only a 50% response. (An above 65% response is seldom observed with either sex attractant or plant attractant formulations.)

Table III. The Epicotyl Volatile Compounds

I_k[a]	M^+	Compound	Fragmentation	Reference	%
		Sesquiterpene hydrocarbons			
1582	204	Caryophyllene	79, 91, 39, 53, 77	16	5.7
1605	204	Humulene	93, 80, 121, 41, 92	16	5.3
1622	204	δ-Guaiene	93, 55, 79, 107, 108	16	1.1
1675	204	( )-Cadinene	119, 105, 91, 81, 161	16	3.3
1685	204	$C_{15}H_{24}$	79, 93, 67, 91, 107		1.1
1700	204	γ-Cadinene	161, 91, 105, 119, 134	16	1.1
		Esters			
1195	168	$C_{10}H_{16}O_2$	69, 101, 88, 67, 59		1.7
1220	172	Methyl nonanoate	74, 87, 43, 41, 55	16	3.2
1475	214	Methyl laurate	74, 55, 87, 57, 59	16	1.1
1675	242	Methyl myristate	74, 55, 87, 143, 199	16	1.9
1870	270	Methyl palmitate	74, 87, 43, 41, 55	16	18.3
2035	284	Unknown	88, 61, 60, 65, 89		5.9
2105	294	Methyl linoleate	74, 87, 43, 41, 95	16	11.0
		Alcohols			
1195	108	Benzyl alcohol	79, 108, 107, 77, 51	16	2.4
1755	222	β-Bisabolol	82, 93, 111, 83, 69	6	11.9
1792	222	Carophyllene alcohol	69, 79, 91, 93, 105	16	1.8
1820	222	Farnesol	69, 81, 67, 68, 93	16	1.3
		Other oxygen-containing compounds			
1420	138	2,4-Dimethyl heptadienal	67, 43, 44, 109, 96	16	1.9
1305	152	α-Pinene oxide	108, 41, 93, 39, 27	16	3.1
1320	152	$C_{10}H_{16}O$	69, 67, 74, 97, 101		3.7
1335	152	$C_{10}H_{16}O$	69, 67, 81, 83, 95		5.3
1455	164	Unknown	55, 69, 81, 136, 135		2.3
1605	206	$C_{14}H_{22}O$	55, 57, 69, 81, 191		1.2
1725	206	( )-Methyl-( )-cyclohexadienyl (2)-furoate	53, 191, 206, 113, 141		
1720	208	( )-Methyl-( )-cyclohexenyl (2)-furoate	53, 193, 208, 113, 141		
1735	208	$C_{14}H_{24}O$	55, 57, 69, 81, 193		1.1
1740	218	$C_{15}H_{22}O$	141, 69, 81, 59, 93		2.2
1750	218	$C_{15}H_{22}O$	69, 59, 83, 74, 111		Trace
1763	220	2,6-Di-*tert*-butyl-*p*-cresol	57, 41, 205, 55, 220	16	1.1
1825	236	$C_{16}H_{28}O$	165, 69, 91, 180, 137		1.1
		Miscellaneous compounds			
1465	135	Benzathiazole	135, 108, 69, 63, 45	16	0.3
1715	185	Unknown	55, 59, 74, 69, 152		1.1
1645	246	$C_7H_6OCl_4$	46, 211, 184, 213, 186		0.3
1785	282	(Pentachloro-ethyl)-cyclohexane	284, 286, 282, 288, 107		0.4
1855	295	( )-Tetrachloro-decylamine	30, 36, 237, 214, 295		0.6

[a] Kovats indices (1961).

The component compounds identified are grouped functionally in Table III with their mass, identity, five most significant fragments in descending order of abundance, literature citation, and percentage content of the total oil.

Table III lists six sesquiterpene hydrocarbons present in the epicotyls of cotton seedlings. Five have been found in cotton buds (Minyard *et al.*, 1966); however, one major sesquiterpene hydrocarbon, *cis*-γ-bisabolene, which is present in cotton buds (2.9% of the total oil) was absent in the epicotyl oil. No monoterpene hydrocarbons were found in epicotyl oil though Minyard *et al.* (1965) reported that they comprise 34.0% of the cotton bud essential oil. However, Croteau *et al.* (1972) reported, as a result of their study of the differential utilization of labeled precursors in peppermint, that monoterpenes and sesquiterpenes are produced by different biosynthetic pathways at separate sites in the plant. Apparently, the epicotyl section of cotton seedlings lacks the ability to synthesize monoterpenes but can synthesize sesquiterpenes.

Table III also lists seven esters found in epicotyl oil. The noticeable presence of the fatty acid esters suggests their importance as high energy food supplies for the meristematic tissues of the tip. The source is undoubtedly the seed. Once conversion of fats to carbohydrates has occurred, photosynthesis then becomes the source of energy, which may explain the apparent absence of fatty acid esters in cotton bud essential oil.

There were four alcohols found in epicotyl oil (Table III). Minyard *et al.* (1968) isolated and identified a new sesquiterpene alcohol, β-bisabolol, which was attractive to the boll weevil. This material accounts for about 5% of the volatiles that can be obtained by steam distillation of cotton buds; it is present in epicotyl oil at a concentration of 11.9%. Of the seven compounds that are present in cotton bud essential oil and are components of the plant attractant complex (Minyard *et al.*, 1969), only two, β-bisabolol and caryophyllene oxide, were found in epicotyl oil. We were particularly interested to determine whether the alcohol fraction of epicotyl oil contained any apparent precursors of the four C_{10} components of the attractant-aggregant pheromone of the male boll weevil (Tumlinson *et al.*, 1969) because males fed fresh cotton are more attractive than those fed artificial diets. Moreover, Tumlinson *et al.* (1970) proposed 2-methyl-6-methylene-2-octen-8-ol as the common precursor of all four components. However, our data provided no support for this possibility. In fact, no C_{10} alcohols were found in the epicotyl essential oil. Of course, some of the oxygen-containing compounds may be alcohols. Also, the alcohol may exist in the pyrophosphorylated state, which may explain why only the epicotyl stimulated feeding in the male though both the epicotyl and hypocotyl were attractive. In this event, nonvolatile components may be primarily responsible for insect preference. The presence of farnesol in epicotyl essential oil is of interest because its pyrophosphate is a key intermediate in terpenoid biosynthesis. Farnesol has not been found in cotton bud essential oil (Hedin *et al.*, 1971b).

A diverse group of oxygen-containing compounds was found in the epicotyl oil. This group contributes heavily to the aroma profile of cotton plants. It may also be a contributing factor in the preference of the boll weevil for the epicotyls of cotton seedlings.

Finally, five miscellaneous compounds were found in the epicotyl oil and listed together. The fourth entry ((pentachloro-ethyl)cyclohexane) is of interest. The cotton seeds used in this study had been treated with the chlorinated fungicide Terraclor (pentachloronitrobenzene and 5-ethoxy-3-(trichloromethyl)-1,2,4-thiadiazole). Indeed, the chlorinated compounds are believed to be metabolic products of Terraclor produced by cotton seedlings. When the essential oil of epicotyls from untreated seed was analyzed, no chlorinated compounds were found.

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