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IDENTIFICATION OF FEEDING STIMULANTS FOR BOLL WEEVILS FROM COTTON BUDS AND ANTHERS[1]

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Abstract—Column chromatography of the pentane extract of freeze-dried cotton buds or anthers yielded a wax–sterol ester fraction that exhibited potent feeding stimulant activity for the cotton boll weevil. The waxes of the wax–sterol ester mixture were responsible for the feeding activity. Saponification of the wax–sterol ester fraction yielded about 15% alcohols and 85% sterols. A $C_{18:1}$ alcohol, dihydrophytol, phytol, and geranylgeraniol constituted 15, 36, 26, and 23%, respectively, of the total alcohols, implicating certain of their long-chain esters as feeding stimulants. Several esters of dihydrophytol, phytol, and geranylgeraniol were identified among the waxes by GC-MS. Certain phytol, geranylgeraniol, and oleyl alcohol esters containing C_{12} to C_{26} acid moieties were synthesized and were found to induce high feeding stimulant activity in the cotton boll weevil.

Key Words—Boll weevil, *Anthonomus grandis*, Coleoptera, Curculionidae, feeding stimulants, cotton buds, anthers, phytol, geranylgeraniol esters, phytol oleate, phytol dodecanoate.

INTRODUCTION

Since Keller et al. (1962) reported the presence of feeding stimulant(s) for boll weevils, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae), in water extracts of cotton squares, considerable work has been done toward the identi-

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fication of compounds that stimulate feeding activity. Hedin et al. (1974) furnished an excellent review of the work on insect feeding stimulants in general, including compounds that stimulated feeding activity of the boll weevil. Several workers reported on a number of compounds from the cotton plant that elicited some degree of feeding in the boll weevil (Hedin et al., 1966; Stuck et al., 1968a, b; Temple et al., 1968). None of the studies, however, have identified compounds of sufficient activity to be considered as the feeding stimulant for the boll weevil. This paper reports on the identification and synthesis of compounds from cotton buds and anthers that are highly active feeding stimulants for the boll weevil.

METHODS AND MATERIALS

Extraction of Cotton Buds and Anthers. Whole cotton buds with bracts were chopped in a blender and then freeze-dried. Anthers were dissected out of buds and then freeze-dried whole. The freeze-dried materials were extracted in a Soxhlet apparatus for 3 hr successively with pentane, ethyl acetate, chloroform, and methanol. The solvents were removed under reduced pressure at 50°C, and the resulting residues were bioassayed.

Feeding-Stimulant Bioassay. Laboratory reared boll weevils, 1 to 4 weeks old, were used in the bioassays. A specific quantity of residue, equivalent to one bud or androecium (aggregate of anthers), of an extract or chromatographic fraction was dissolved in 1 ml of hexane or methanol. For bioassay of the synthetic esters, a 0.04 M solution in hexane was prepared. Approximately 20 μl of each test solution was placed within two of four 1-cm-diameter circles drawn around the periphery of 7-cm-diameter qualitative grade filter paper. Similarly, solvent blank was applied to the two remaining 1-cm circles. After the solvent evaporated, the filter paper was placed inside an 11-cm-diameter Petri dish on a 0.5-cm layer of 2.5% agar. Ten insects were placed into each dish, and the dishes were placed in a dark environment overnight at 29°C. Feeding response was determined by totaling the number of feeding punctures within the treated circles then subtracting the total number of punctures in the blank. Twenty replicas were obtained.

Chromatography. The residue from the pentane extractions was chromatographed over 25 g of hexane-washed Merck silica gel (2.5 × 13 cm), and the column was eluted with 100-ml volumes of 0, 3, 10, 25, and 50% ether in hexane. Of the 3% ether in hexane eluent, fractions of 75-ml and 25-ml volume were collected. TLC analyses were conducted on silica gel plates. The solvent systems were toluene–hexane (1 : 1) and toluene–ethyl acetate (9 : 1).

Feeding Stimulant Analyses by GLC and GC-MS. Natural and synthesized feeding stimulants were analyzed by GLC and GC-MS. GLC was performed with a Varian model 3700 gas chromatograph equipped with a 15-m × 0.32-

mm DB-1 fused silica capillary column (0.25 μm film). Mixtures of esters were injected onto the column at 240°C, programed, after an initial hold of 1 min, 10°/min to 285° and held; total time 40 min. For the alcohols, the program was 1 min at 70°C, 10°/min to 285°C and held for 20 min. GC-mass spectral data were obtained with a Finnigan model 4500 spectrometer fitted with a 15-m × 0.25-mm DB-1 fused silica capillary column (0.1 μm film thickness) and connected to an Incos data system. Mixture of esters or alcohols were injected at 240°C, held for 1 min, then programed at 10°/min to 290°C and held as in the capillary GLC analyses. The compounds were analyzed by ammonia chemical ionization and electron impact mass spectrometry.

Saponification of Esters. The esters were saponified by refluxing with 5% potassium hydroxide in methanol–benzene (5:1) for 4 hr. Most of the solvent was removed under vacuum and the mixture was diluted with water, acidified with dilute hydrochloric acid, and extracted three times with hexane. The hexane solution was washed with water, dried over sodium sulfate, and the hexane removed under vacuum. The dried residue was dissolved in ether and treated with an excess of diazomethane to react with fatty acids. The ether was removed under vacuum, and the residue was chromatographed.

Synthetic Esters. The esters listed in Table 3 were prepared via reaction of the acid with an excess of thionyl chloride in benzene at reflux temperature. After removal of solvent and excess thionyl chloride, the acid chloride was redissolved into dry benzene and added to a gram equivalent of the alcohol and pyridine in dry benzene. The reaction mixture was heated overnight at 60°C, then solvent was removed under vacuum and the dry residue was triturated with hexane. The hexane-soluble material was chromatographed over activity grade II alumina and eluted with hexane and 5%, and 10% ether in hexane. The fractions were analyzed by TLC, and the fractions showing only one component were further analyzed by capillary GLC before the purified ester fractions were combined.

RESULTS AND DISCUSSION

Extracts of Buds and Anthers. When cotton buds or anthers were extracted with pentane, ethyl acetate, chloroform, and methanol, the pentane extracts of either buds or anthers elicited the highest feeding response. The methanol extract of anthers was also active (Table 1). The pentane and methanol extractions of 25 g of freeze dried buds (193) yielded 0.69 g and 4.72 g, respectively, whereas similar extractions of 25 g of freeze-dried anthers (1000 androecia) gave 1.96 g and 6.37 g, respectively. The great differences in the polarities of pentane and methanol indicate that there are structurally different feeding stimulants for the boll weevil in cotton buds and anthers. Our results only deal with those identified from the pentane extracts.

TABLE 1. RESPONSE OF BOLL WEEVILS PRESENTED CRUDE EXTRACTS OF COTTON BUDS AND ANTHERS[a]

	Number of feeding punctures	
Extracts	Buds	Anthers
Pentane	92	157
Methanol	17	88
Blank	12	3

[a]The quantity of mass applied to filter paper from the pentane and methanol extracts from buds were 72 and 489 μg, respectively; from anthers 39 and 127 μg respectively.

Chromatography of Pentane Extract and Identity of Active Fraction. When pentane extracts from cotton buds or anthers were chromatographed and the fractions bioassayed, fraction 2, which was eluted with the 75-ml volume of 3% ether in hexane and contained only trace quantity of mass, was inactive; however, the material eluted with the last 25-ml volume of 3% ether in hexane (fraction 3) showed high feeding stimulant activity (Table 2). These fractions contained approximately 20% and 22.5% of the total residue from the pentane extract of cotton buds and anthers, respectively. The materials had TLC R_f's similar to those of authentic waxes and sterol esters. Infrared analyses of the materials also supported the structures of waxes and sterol esters.

Indirect Identification of Waxes as Feeding Stimulants. Since it was not possible to physically separate the waxes from sterol esters in sufficient quantities for testing each component for feeding stimulant activity, the wax-sterol ester mixture was saponified. The aliphatic alcohols and sterols were separated into an alcohol-4,4-dimethylsterol fraction and a desmethylsterol fraction. Reconversion of each fraction to a mixture of esters consisting of C_{14} and C_{16} acid

TABLE 2. RESPONSE OF BOLL WEEVILS PRESENTED CHROMATOGRAPHIC FRACTIONS OF PENTANE EXTRACTS OF COTTON ANTHERS[a]

Fractions	Number of feeding punctures (anthers)
1	22
2	20
3	403
4	22
5	24
6	24

[a]The quantity of material in fraction 3 applied to the test filter was 9 μg.

moieties (tetradecanoic and hexadecanoic acids were two of the major fatty acids of the saponified wax–sterol ester mixture) followed by feeding stimulant assays showed that the esters of the alcohol-4,4-dimethylsterol fraction were active. A later assay of the C_{14} and C_{16} ester mixture of the purified 4,4-dimethylsterol was found to be inactive, indicating that the waxes of the wax-sterol ester mixture were responsible for the feeding stimulant activity.

Identities of Alcohol Moieties of Waxes. Analyses by capillary GLC of the alcohol–sterol mixture obtained from the saponification of the mixtures of waxes and sterol esters from cotton buds or anthers were quite similar and indicated that the aliphatic alcohol and sterol contents represented 15 and 85%, respectively. There were essentially only four aliphatic alcohols, and analysis by CI-MS and EI-MS of the alcohols with retention times of 11.92, 12.15, 12.45, and 13.08 min and representing approximately 15, 36, 26, and 23%, respectively, of the total nonsteroidal alcohols, indicated molecular masses of 268, 298, 296, and 290. They were identified as a C_{18} alcohol with a double bond, dihydrophytol, phytol, and geranylgeraniol, respectively. Authentic (Z)-9-oleyl alcohol, dihydrophytol, phytol, and geranylgeraniol had retention times of 11.96, 12.22, 12.5, and 13.13 min, respectively. The electron impact and ammonia chemical ionization mass spectra of the authentic alcohols and alcohols from cotton buds or anthers were identical. The retention times of sterols were from 19 to 23 min.

Analyses of Waxes of Wax–Sterol Ester Fraction by GLC and GC-MS. The active wax–sterol ester fraction from cotton buds or anthers, when analyzed by capillary GLC, gave similar chromatograms, although the chromatogram of anthers exhibited fewer minor peaks. Each showed several major peaks of waxes with retention times of 4–9 min (Figure 1A) and several minor peaks with retention times of 9–12.5 min (some sterol esters). The remaining mass, representing approximately 80% of the total quantity injected, was eluted in 15.9–37 min and consisted predominantly of sterol esters as indicated by mass spectral analyses.

The ammonia CI GC-MS total ion monitor trace (Fig. 1B) is quite similar to the capillary GLC. All peaks, when analyzed by ammonia CI, gave $(M + NH_4)^+$ adduct ions as base peak or of high intensity. Esters of phytol and geranylgeraniol were readily identified by ammonia CI mass spectra which exhibited in addition to $(M + NH_4)^+$ adduct ions, $M + NH_4 - RCO_2H$ fragments at m/z 296 and 290 which are, coincidentally, equivalent to the molecular weights of phytol and geranylgeraniol, respectively. The $(M + NH_4)^+$ adductions and the $(M + NH_4 - RCO_2H)^+$ ions of high abundances indirectly indicate the molecular weights of the ester, phytol, geranylgeraniol, and the acid moiety. Additionally, the esters of geranylgeraniol gave $M + H - RCO_2H$ fragments at m/z 273.

Thus, even though the corresponding esters of phytol and dihydrophytol were poorly separated by GLC, the phytol esters containing C_{16}, $C_{18:1}$, C_{22}, and C_{24} acid moieties were identified in peaks 2, 4, 6, and 7, respectively (Figure

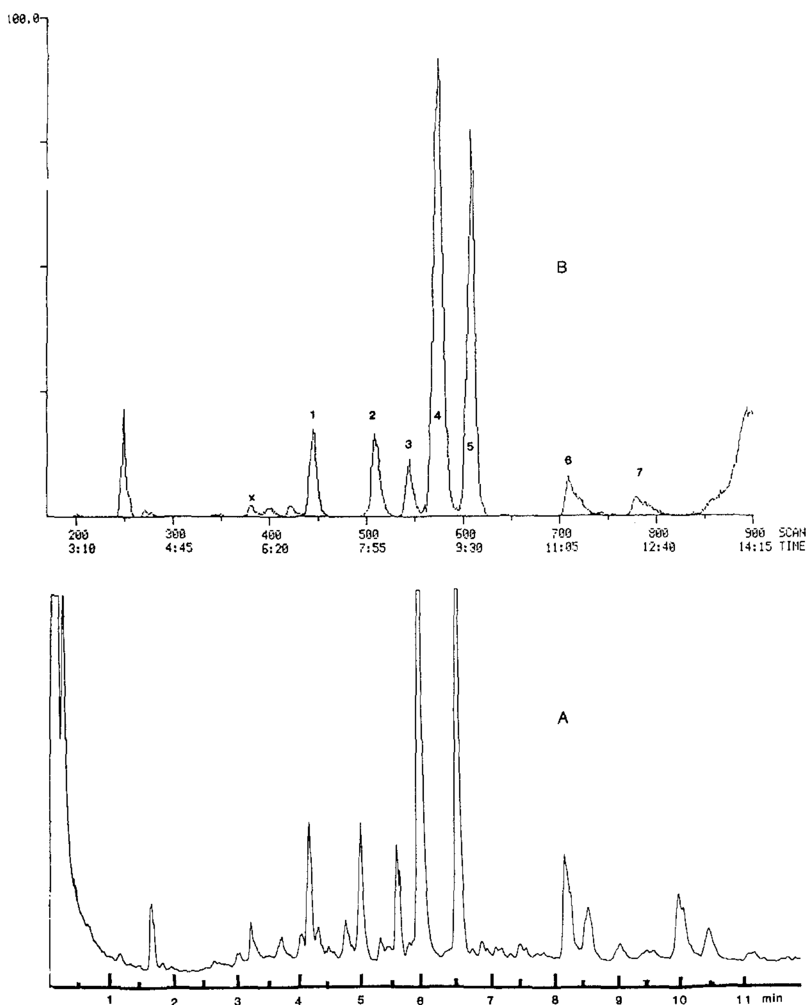

FIG. 1. (A) Capillary GLC trace of the wax region of the wax–sterol ester fraction of cotton anthers; (B) ammonia CI-MS total ion monitor trace of the wax region of the wax–sterol ester fraction of cotton anthers.

1B). They eluted on the back side of these peaks. The molecular weights (by mass spectral analyses) of these esters were 534, 560, 618, and 646, respectively. At the beginning of the front side of the region where some of sterol ester began to elute, the phytol ester (mol wt 674) containing a C_{26} acid moiety was also observed. Since molecular weights of the corresponding esters of dihydrophytol are two mass units higher than those of phytol, the molecular weights of 536 and 562 for the esters eluting on the front side of peaks 2 and 4 suggest

that these are dihydrophytol esters containing C_{16} and $C_{18:1}$ acid moieties, respectively. Similarly, the major esters of peaks 6 and 7 are dihydrophytol esters containing C_{22} and C_{24} saturated acid moieties, respectively. It should be mentioned that saponification of the wax–sterol ester fraction yielded predominantly even-numbered saturated straight chain acids from C_{12} to C_{28}. The C_{14} to C_{28} acids were about equally distributed.

Interestingly, the geranylgeraniol esters eluted as peaks of high purity without observable contamination. The adduct $(M + NH_4)^+$ ion, and the $M + NH_4 - RCO_2H$ and $M + H - RCO_2H$ fragments readily identified peaks 3 and 5 as geranylgeraniol esters containing C_{16} and $C_{18:1}$ acid moieties, respectively. The molecular weights of these two esters were 528 and 554.

Other esters were observed in peaks 1, 2, and 4. Ammonia CI GC-MS analyses of peak 1 showed material of a molecular weights of 486 and a small quantity of mol wt of 484; peak 2 showed mol wt of 506 and peak 4 showed mol wt of 532. We could not identify these esters or speculate as to their identity since the alcohol or fatty acid moieties of these peaks could not be determined by EI- or CI-mass spectral analyses. EI mass spectral analyses were of little diagnostic value for the esters.

Synthetic Esters. Since our results indicated that the reconversion of the alcohols to esters restored activity, we synthesized and bioassayed a series of fatty acid esters of oleyl alcohol (not necessarily the $C_{18:1}$ alcohol present in anthers or buds), dihydrophytol, phytol, and geranylgeraniol including those found in the wax–sterol ester fraction. The synthetic esters corresponding to those found in the wax mixture from cotton buds or anthers exhibited similar retention times by GLC and gave, by ammonia CI, spectra similar to those of the naturally occurring esters.

In our bioassay, a compound was rated as having high feeding stimulant activity if it induced feeding and caused weevils to produce 50 or more punctures in the treated area of the filter paper than in blank or untreated areas. Our results thus indicate that a number of synthetic phytol and geranylgeraniol esters have high feeding stimulant activity when tested below the 500 μg level (Table 3). Higher quantities of material on the test paper increased feeding stimulant activity. The phytol ester containing a C_{12} acid moiety was the most active of the phytol esters tested. Yet, GC-MS analyses (Figure 1B, peak X) indicate that this ester is present only in a small quantity in cotton buds or anthers.

Peak 5 (Figure 1B), which was identified as a geranylgeraniol ester containing a $C_{18:1}$ acid moiety and subsequently identified as geranylgeraniol oleate, is a major component of the waxes of cotton buds and anthers. The synthetic geranylgeraniol oleate, however, did not exhibit high feeding stimulant activity.

The dihydrophytol esters containing the C_{14} and C_{16} acid moieties were prepared and tested, and they were inactive. Of the oleyl alcohol esters tested, only the ester containing a C_{22} acid moiety showed high activity. Eicosanol ester with a C_{14} acid moiety was inactive in the feeding stimulant bioassay. Similarly,

TABLE 3. FEEDING RESPONSE OF BOLL WEEVILS PRESENTED SYNTHETIC ESTERS[a]

Alcohol moiety	Acid moiety	Feeding response
Geranylgeraniol	C_{12}	32
	C_{14}	16
	C_{16}	31
	C_{18}	−4
	$C_{18:1}$	12
	C_{20}	75
	C_{22}	110
Phytol	C_{10}	15
	C_{12}	110
	C_{14}	53
	C_{16}	36
	C_{18}	73
	$C_{18:1}$	32
	C_{20}	59
	C_{22}	54
	C_{26}	59

[a] The quantities presented in application of 20 μl of 0.04 M solution ranged from 378 to 490 μg and 361 to 540 μg for the esters of geranylgeraniol and phytol, respectively.

farnesol with a C_{18} acid moiety was inactive. The results suggest that an allylic diterpene alcohol moiety is a prerequisite for an ester to be active, although phytol phytanoate was inactive. Increasing the chain length of the geranylgeraniol esters appears to enhance activity.

A mixture of synthetic esters of phytol and geranylgeraniol found in the wax–sterol fractions of buds and anthers did not show activity higher than that of the most active component present in the mixture. Certain of the synthetic esters, for example the phytol esters containing C_{12}, C_{18}, and C_{26} acid moieties, were more active than the wax–sterol ester fraction of anthers or buds when tested at concentrations approximating that of the waxes in the extracts. For example, 20 μl of 0.005 M solution or 56 μg of the phytol ester with the C_{18} acid moiety elicited a feeding response of 80 punctures compared to three punctures in the blank. The ratio of response by weevils presented 1.75 μg and 0.877 μg of this ester to blank was 19:5 and 26:6, respectively. Thus, the low threshold level of feeding response is below 1 μg for this compound.

Host-plant selection by phytophagous insects in early infestation stages is often partially dependent on plant surface stimuli, e.g., stimuli that influence aphids while walking and probing, and beetles during the first probe (Klingauf et al., 1978). The alkane fraction of the host plant promoted pea aphid, *Acyrthosiphon pisum*, movement from the upper to the lower leaf side. It has been

shown that, at least in many cases, the chemical nature of the surface is important in selections. The outer surfaces of the leaf of all terrestrial angiosperms and gymnosperms are covered by a cuticle some 1–13 μm thick. The commonest components are *n*-alkanes, *n*-primary alcohols, fatty acids, and wax esters (Chapman, 1977), although many other chemicals are recorded as minor constituents. The alcohols, *n*-hexacosanol and *n*-octacosanol, have been recently identified from leaves of the mulberry as potent feeding stimulants for larvae of the silkworm, *Bombyx mori* (Mori, 1982). Because *n*-tetracosanol and *n*-triacontanol are also present in mulberry leaves but did not stimulate larval feeding, the author concluded that the chemoreceptors for food selection of the silkworm are precise in recognizing the carbon skeleton of chemicals.

The fact that many esters of phytol and geranylgeraniol have such high feeding stimulant activity certainly suggests that these compounds in cotton buds and anthers are the major feeding stimulants for the cotton boll weevil. The identical carbon chain lengths of phytol and geranylgeraniol also suggest that the chemoreceptor of the boll weevil may be stimulated by the carbon skeleton of these alcohols. We do need to determine, however, the structure of the $C_{18:1}$ alcohol derived from the waxes, and whether the sterol esters present in the wax–sterol fraction enhance the feeding activity of the long-chain esters of phytol and geranylgeraniol.

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