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Pesticide Effects on the Plant Cuticle: III. EPTC Effects on the Qualitative Composition of *Brassica oleracea* L. Leaf Cuticle[1]

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Abstract. **S-Ethyl dipropylthiocarbamate (EPTC, 2.24 kg/ha) altered wax composition on developing leaves of cabbage [*Brassica oleracea* L. (Capitata group) cv. Market Prize], but did not affect cutin composition. The alkane, ketone and secondary alcohol content of the epicuticular wax was reduced and ester content increased. C_{29} constituents (alkane, ketone, aldehyde and *sec*-alcohol) accounted for 72.5% (34.1 $\mu g/cm^2$) and 40.2% (7.2 $\mu g/cm^2$) of the epicuticular wax on control and EPTC-treated leaves respectively. Homlog composition within a chemical group was not changed. Chemical composition was similar for abaxial and adaxial leaf surfaces, and the EPTC-induced change in chemical composition was similar for both surfaces. In contrast with epicuticular wax, cuticular wax contained higher percentages of fatty acids and primary alcohols, and lower percentages of alkanes, and ketones. All constituents except the unidentified polar materials and fatty acids were lower in cuticular wax extracted from EPTC-treated than non-treated plants. The main component of the cutin fraction from both control and EPTC-treated plants was identified as dihydroxyhexadecanoic acid. Cutin acids were not quantitatively changed by the EPTC treatment.**

Several pesticide chemicals inhibit leaf cuticle development resulting in greater plant sensitivity to the external environment. Plants treated with these chemicals are often more susceptible to fungal attack (10) or foliar applied herbicides (5, 10, 20), lose water at greater rates, or retain greater quantities of an aqueous spray than nontreated plants (3, 5, 6, 10, 14, 20). The increased plant response has been associated with reduced levels of epicuticular wax and/or fine-structure. Certain thiocarbamates cause marked changes in the quantity, quality, and surface fine-structure of epicuticular wax on developing leaves of pea, cabbage, and sicklepod, but little is known of their effects on the qualitative composition of other cuticular components, or how these alterations may be related to cuticular permeability (3, 4, 8, 9, 10, 17, 24, 25, 26, 27).

We have initiated a study designed to establish the effects of pesticide chemicals, principally EPTC, on cuticle development and to utilize cuticles so altered in gaining a better understanding of cuticular permeability. Earlier we reported on the nature and duration of the EPTC effect on the cuticle as indexed by epicuticular wax production (8) and the effects of EPTC upon the morphology and quantitative composition of the cuticular membrane (9). We now further characterize the effects of EPTC on the chemical composition of cabbage leaf cuticle.

Materials and Methods

Plant culture and cuticle fractionation. Cabbage plants were grown, and treated with EPTC, 2.24 kg/ha, as reported previously (8). Briefly, plants were treated with EPTC as an aqueous soil drench (10 ml/pot), when in the 4-6 leaf stage. The youngest visible node was marked, and the leaves were harvested for experimental studies from the marked node, usually number 7, 14-21 days after chemical treatment. Leaves (10-15) for analysis were harvested from 3-5 plants per treatment, each treatment replicated 3 times. Epicuticular wax, isolation of cuticular membranes, cuticular wax, and the carbonate soluble materials were extracted from the cutin matrix as previously described (9). The cutin matrix (about 50 mg) was refluxed for 3 hr with 25 ml 3% (wt/vol) sodium methanol (sodium methoxide). The reaction mixture was filtered and the residue refluxed for an additional 20 min. The combined methanolic filtrates were acidified with 2M H_2SO_4 (25 ml, 10% vol/vol H_2SO_4:methanol) and taken to dryness on a rotary evaporator. The residue was suspended in 50 ml distilled water and the methylated cutin acids were extracted with chloroform. This method yields esters of acids originally esterified in the cutin polymer and prevents the formation of methoxy methyl ester artifacts (13). Using the method of Eglington et al. (7), trimethylsilyl (TMS) esters were prepared using N,O-bis-(trimethylsilyl) acetamide.

Thin layer chromatography. The constituents of epicuticular and cuticular waxes were separated by TLC, and general chemical classes were identified by comparison with standards, or published Rf values (1, 21, 22). Waxes were dissolved in chloroform (10 mg/ml, wt/vol) and spotted (5μl) on precoated silica gel G thin-layer plates (Uniplate, 250 μm, Analtech, Inc., Newark, Delaware), which were prewashed in redistilled benzene and dried at 110°C for 30 min. Spotted plates were developed in benzene, and wax constituents were localized by charring (160°C) after spraying with H_2SO_4, or by reacting with iodine vapor. For quantitative TLC, epicuticular wax (20 mg) from control and EPTC-treated plants was streaked as a narrow band (2 mm) on each of 10 thin-layer plates, developed, localized with iodine vapor, and recovered by refluxing the TLC fraction with chloroform for 2 hr. After filtration and evaporation of the solvent, each fraction was weighed and analyzed by GLC.

Gas liquid chromatography. All GLC data were obtained using a Packard 7300-gas liquid chromatograph equipped with a hydrogen flame ionization detector and a temp programmer. The column was stainless steel (2 mm I.D. × 1.8 m length) packed with Chromosorb W (80/100 mesh), coated with SE-30 (1.25%). Operating conditions were: nitrogen flow 40 ml/min, inlet and detector temp 360°C, and column temp programmed from 120 to 350°C at 6° per min. Fatty acids were methylated using diazomethane (23), all other chemical groups were detected without conversion to a derivative. Unknowns were identified by comparing elution times with those of known standards. Standard curves were constructed from: n-alkanes

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C_{22}, C_{24}, C_{28}, C_{32}, C_{36} (Analabs); primary alcohol C_{22} (J. T. Baker Co.); C_{26}, C_{28} (Analabs); methyl esters of fatty acids C_{12}, C_{14}, C_{16}, C_{18}, C_{20}, C_{22} (Packard Instruments); aldehydes C_{18} (Analabs); C_{24}, C_{26}, C_{28} (isolated from *Chenopodium album* L.); ketone C_{35} (J. T. Baker Co.); secondary alcohol C_{29} (isolated from *Brassica oleracea*); esters C_{32}, C_{36} (Analabs); C_{46} (gift from A. P. Tulloch, Prairie Regional Lab., Saskatoon, Saskatchewan); C_{40}, C_{42} (synthesized). Relative retention (T_{rel}) data were determined by comparing unknown and unidentified elution times with elution times of internal standards: n-tetracosane for whole wax, alkanes, ketones, and secondary alcohols; 1-docosanol for primary alcohols; methyl docosanate for methyl esters of fatty acids; and octadecyl stearate for long chain esters. Quantification was determined by peak areas (height × base at 1/2 peak height). Corrections were made for detector response based on n-octacosane. All date are expressed as the mean of a minimum of 3 determinations per sample.

Cutin acid identification. Cutin acids were converted to TMS ether methyl esters and were separated by GLC using the same column and operating conditions as for wax analysis, except temp was programmed from 120° to 280°C at 5° min, and inlet and detector temp was maintained at 290°. Unknowns were compared with TMS ether methyl esters of hexadecanoic acid, ω-hydroxypentadecanoic acid, ω-hydroxyhexadecanoic acid, dihydroxyhexadecanoic acid, 9, 10, 16-trihydroxyoctadecanoic acid (gift, E. A. Baker, Long Ashton Research Sta., UK). Identification was confirmed by GC-MS utilizing a LKB-9000 GC-MS, interfaced with a PDP 8/I computer. Instrument conditions were: SE-30 (1%) column, temp programmed (120-180°) at 5°/min, ion source 70.0 eV. Unidentified constituents were expressed in terms of T_{rel} for ω-hydroxyhexadecanoic aicd.

Results

Wax composition-qualitative TLC. Cabbage epicuticular and cuticular waxes were clearly resolved by TLC into their major chemical classes (Fig. 1). Epicuticular wax composition was similar for adaxial and abaxial surfaces. Comparisons of epicuticular and cuticular waxes indicated marked differences in composition due to EPTC-treatment. EPTC had a similar effect on chemical composition of epicuticular wax for both surfaces. Marked decreases were noted in the alkane, ketone, and *sec*-alcohol fractions; an increase in the ester fraction occurred due to EPTC-treatment.

Epicuticular wax composition. EPTC (2.24 kg/ha) caused a 61.7% reduction in total epicuticular wax deposition (Table 1).

Table 1. Chemical composition of epicuticular wax isolated from developing leaves of control and EPTC-treated cabbage plants.

Chemical fraction	Treatment[z] Control (% of total)	EPTC (% of total)	Control (μg/cm²)	EPTC (μg/cm²)	Change (μg/cm²)
Alkane	26.0	18.9	12.2	3.4	− 8.8
Ester	8.3	34.5	3.9	6.3	+ 2.4
Ketone	35.9	19.4	16.9	3.5	−13.4
Aldehyde	5.5	6.0	2.6	1.1	− 1.5
Secondary alcohol	10.7	3.0	5.0	0.5	− 4.5
Ketol	2.1	1.8	1.0	0.3	− 0.7
Primary alcohol	7.6	11.6	3.6	2.1	− 1.5
Fatty acid	3.9	4.8	1.8	0.8	− 1.0
Total[y]	100.0	100.0	47.0	18.0	−29.0

[z]Identification and quantification by TLC.

[y]Mean separation for total wax between control and EPTC treatment significantly different by Tukey's ω-test, 5% level.

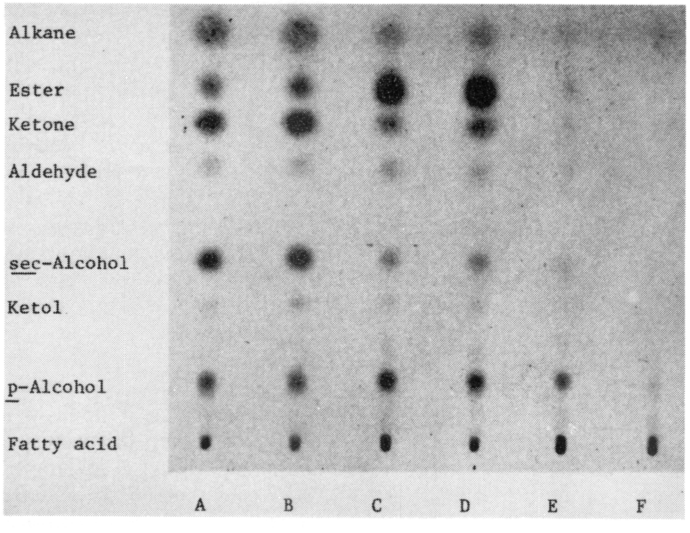

Fig. 1. Thin-layer chromatogram of epicuticular and cuticular waxes extracted from developing leaves of control and EPTC-treated plants illustrating wax fractions: A: epicuticular, control, adaxial surface. B: epicuticular, control, abaxial surface. C: epicuticular, EPTC, adaxial surface. D: epicuticular, EPTC, abaxial surface. E: cuticular (both surfaces), control. F: cuticular (both surfaces), EPTC.

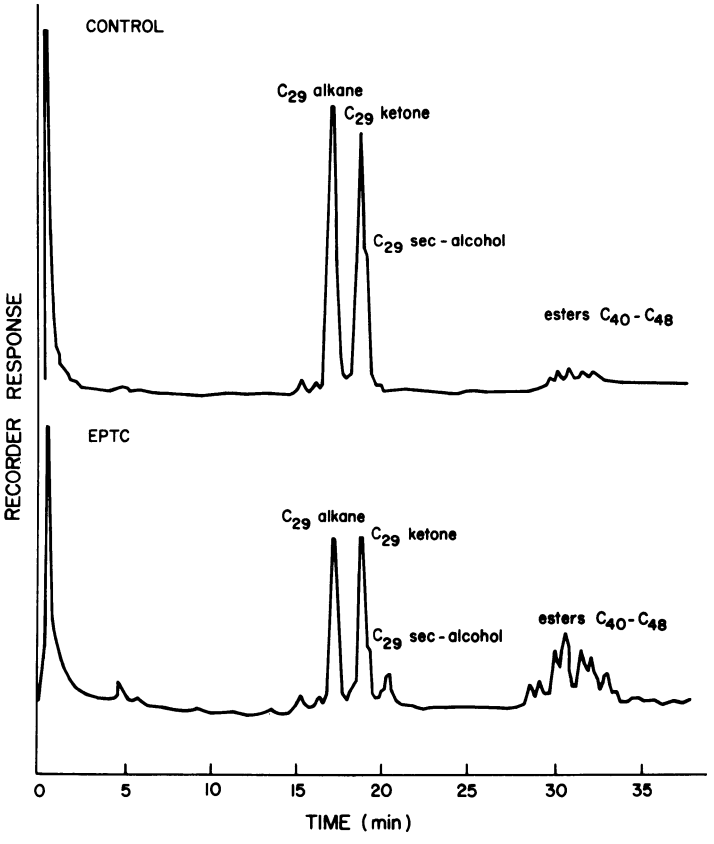

Fig. 2. GLC-traces of epicuticular wax isolated from developing leaves of control and EPTC-treated cabbage plants. Column SE-30 (1.25%), programmed from 120 to 350°C at 6°/min.

Table 2. Homolog composition of epicuticular wax isolated from developing leaves of control and EPTC-treated cabbage plants.[z]

Chemical Class	Carbon number	T_{rel}	Control (% of total)	EPTC[y] (% of total)
Alkane	27		tr[x]	1.0
	28		0.1	0.1
	29		25.0	17.5
	30		0.1	0.1
	31		0.6	0.1
Ester	40 - 47		8.3	34.5
Ketone	29		35.9	19.3
Aldehyde	26		1.3	1.3
	27		0.8	0.5
	28		1.4	3.0
	29		0.9	0.6
	30		1.1	0.7
Secondary alcohol	29		10.7	2.8
Ketol[w]			2.2	1.8
Primary alcohol[v]	24	1.19	0.5	0.9
	26	1.31	2.3	6.3
		1.39	1.7	--[t]
	28	1.49	1.3	1.6
		1.58	1.1	1.1
		1.61	0.8	1.5
Fatty acid[u]	14	0.30	0.2	0.3
	15	0.38	--[t]	0.2
	16	0.47	0.1	0.1
		0.67	--[t]	0.1
	22	1.00	0.1	0.3
		1.17	0.2	0.1
		1.24	0.1	0.1
	26	1.33	1.3	0.5
		1.42	0.1	0.1
	28	1.48	1.4	1.3
		1.56	0.1	0.5
	30	1.63	0.3	1.0
		1.75	--	0.7

[z]Determined by GLC.

[y]Control = 47.0 $\mu g/cm^2$; EPTC-treated = 18.0 $\mu g/cm^2$.

[x]Trace amount, quantity not determined.

[w]Identified by TLC Rf.

[v]T_{rel} based on 1-docosanol.

[u]T_{rel} based on methyl docosanate.

[t]Not detected.

Table 3. Chemical composition of cuticular wax isolated from developing leaves of control and EPTC-treated cabbage.[z]

Chemical fraction	Control (% of total)	EPTC (% of total)	Control ($\mu g/cm^2$)	EPTC ($\mu g/cm^2$)	Change ($\mu g/cm^2$)
Alkane	18.7	9.2	1.31	0.87	−0.44
Ester	6.3	1.7	0.44	0.16	−0.28
Ketone	15.8	1.6	1.11	0.15	−0.96
Aldehyde	2.4	tr[y]	0.17	tr[y]	−0.17
Secondary alcohol	6.9	tr[y]	0.48	tr[y]	−0.48
Primary alcohol	20.5	6.6	1.44	0.63	−0.81
Fatty acid	24.9	61.8	1.74	5.87	+4.13
Unidentified polar fraction	4.5	19.1	0.32	1.82	+1.50
Total[x]	100.0	100.0	7.0	9.5	+2.50

[z]Determined by GLC.

[y]Trace amounts not used in calculation of $\mu g/cm^2$, or percent of total.

[x]Mean separation for total wax between control and EPTC treatment not significantly different by Tukey's ω-test, 5% level.

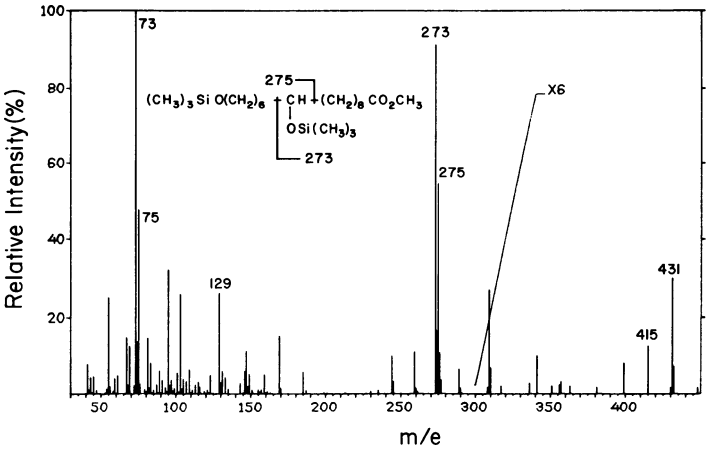

Fig. 3. Mass spectra of the dihydroxyhexadecanoic acids (TMS ether methyl ester), isolated from cutin of *Brassica oleracea*.

All chemical constituents (of the epicuticular wax) were not affected equally. The alkane (−8.8 $\mu g/cm^2$), ketone (−13.4 $\mu g/cm^2$), and secondary alcohol (−4.5 $\mu g/cm^2$) fractions were most notably reduced, with minor decreases in the fatty acid (−1.0 $\mu g/cm^2$), primary alcohol (−1.5 $\mu g/cm^2$), ketol (−0.7 $\mu g/cm^2$), and aldehyde (−1.5 $\mu g/cm^2$) fractions. Long chain esters increased (+2.4 $\mu g/cm^2$) as a result of EPTC-treatment. When expressed as a percentage of total epicuticular wax, the ester fraction becomes the most dominant constituent (34.5% compared to 8.3% in the control).

Separation of the epicuticular wax into major chemical groups was accomplished by GLC. The major alkaline (C_{29}), ketone (C_{29}) and long chain esters were clearly resolved when temp was programmed from 120-340°C at 6°C per min. The secondary alcohol (co-chromatographed with the ketone) appeared as a shoulder on the C_{29} ketone peak (Fig. 2). Primary alcohols and aldehydes co-chromatographed with the alkanes and ketones, but, because of their small quantities, and low detector responses, contributed little to peak areas.

Table 4. Composition of cutin isolated from developing leaves of control and EPTC-treated cabbage plants.

Chemical constituent		Control (% of total)	EPTC (% of total)	Control ($\mu g/cm^2$)	EPTC ($\mu g/cm^2$)	Change ($\mu g/cm^2$)
Hexadecanoic acid		5.9	5.2	2.42	2.00	−0.42
Octadecanoic acid		2.0	1.8	0.82	0.69	−0.13
ω-Hydroxyhexadecanoic acid		10.2	7.4	4.18	2.85	−1.33
10,16-Dihydroxyhexadecanoic acid		58.9	49.4	24.15	19.02	−5.13
9,16-Dihydroxyhexadecanoic acid		6.1	6.5	2.50	2.50	−0.00
8,16-Dihydroxyhexadecanoic acid		1.4	3.3	0.57	1.27	+0.70
Unknown T_{rel}[z]	1.04	5.1	--[y]	2.09	--[y]	−2.09
	0.92	10.4	9.2	4.26	3.54	−0.72
	0.83	--[y]	13.7	--[y]	5.27	+5.27
	0.46	--[y]	3.4	--[y]	1.31	+1.31
Total[x]		100.0	100.0	41.0	38.5	−1.5

[z]Based on ω-hydroxyhexadecanoic acid.

[y]--not detected.

[x]Mean separation between control and EPTC treatment not significantly different by Tukey's ω-test, 5% level.

Table 5. Major fragments and relative intensity of mass spectra of derivatized TMS ether methyl ester of 10,16-dihydroxyhexadecanoic acid isolated from cutin of *Brassica oleracea* leaves of nontreated plants.[z]

m/e	Fragment	Relative intensity
73	$(CH_3)_3Si$	100
75	$(CH_3)_2SiOH$	48
89	$(CH_3)_3SiO$	4
129	$CH_2 = CHCHOSi(CH_3)_3$	27
273	$CH_3CO_2(CH_2)_8CHOSi(CH_3)_3$	33
275	$(CH_3)_3SiO(CH_2)_6CHOSi(CH_3)_3$	52
415	$M - OCH_3$	3
431	$M - CH_3$	
446	$(CH_3)_3SiO(CH_2)_6CH(CH_2)_8CO_2CH_3$ $OSi(CH_3)_3$	--[x]

[z]Mass sprectra of major cutin constituent isolated from EPTC-treated plants was identical.

[x]Not detected.

Comparison of epicuticular wax from control and EPTC-treated plants, from several experiments, and from abaxial and adaxial surfaces confirmed the TLC data.

EPTC-treatment did not alter the homolog composition of epicuticular wax within a chemical class (Table 2). There was a marked reduction of C_{29} constituents. The C_{29} constituents accounted for 40.2% (7.2 $\mu g/cm^2$) of the epicuticular wax in EPTC-treated plants compared with 72.5% (34.1 $\mu g/cm^2$) for the controls (Table 2).

Cuticular wax composition. There were no significant quantitative differences in the cuticular wax between control (7.0 $\mu g/cm^2$) and EPTC-treated (9.5 $\mu g/cm^2$) plants (Table 3). Cuticular wax from EPTC-treated plants was lower in all chemical constituents, except for fatty acids, and unidentified polar fraction, which were increased when compared to cuticular wax isolated from control plants (Table 3). When expressed on a percentage basis, regardless of treatment, cuticular wax contained a higher percentage of polar materials (fatty acids, primary alcohols, secondary alcohols) than epicuticular wax (Tables 2, 3).

Cutin composition. EPTC-treatment did not affect cutin acid composition. The major cutin constituent (58.9% for control; 49.4% for EPTC-treated) was identified by GLC and confirmed by GC-MS as 10,16-dihydroxyhexadecanoic acid (Fig. 3, Table 5), with lesser amounts of 8,16 and 9,16-dihydroxyhexadecanoic acids, hexadecanoic acid, octadecanoic acid and ω-hydroxyhexadecanoic acid (Table 4). 10,16-Dihydroxyhexadecanoic acid was identified by comparing mass sprectra with previously published spectra (7, 13).

Discussion

EPTC altered the quantitative and qualitative composition of epicuticular wax on developing cabbage leaves. While the chemical composition of the epicuticular and cuticular waxes was modified with EPTC, no significant change was observed in the constituents of the cutin. The primary effect of EPTC was on the wax fractions and therefore, because of the critical role of epicuticular wax (18, 19) on wettability and permeability of the cuticular membrane, EPTC may affect the cuticle's efficiency as a protective covering.

Current concepts (16) on biosynthesis of surface wax in *Brassica* suggest that long chain constituents (greater than C_{16}) are formed by addition of acetate units to existing fatty acids until a chain length of 30 carbons is attained, followed by decarboxylation to the major alkane, n-nonocosane. The latter is further oxidized to form the major secondary alcohol, and ketone constituents, nonocoson-14-ol, nonocoson-15-ol, and nonocoson-15-one (15). The aldehyde and primary alcohol chain lengths are similar (C_{26}, C_{28}) and are of sufficient length to have been derived by the reduction of fatty acids via the fatty acid elongation pathway. The reaction of free primary alcohols with endogenous fatty acids to form esters is probably enzyme mediated (15). The free primary alcohol chain length is similar to that of the alcohol found in the wax ester which suggests a common origin (16), while variation in the fatty acid moiety of the ester indicates an origin from different sources (16).

Our data suggest that deposition of all chemical classes of the epicuticular wax fraction of the cuticle except esters were inhibited by EPTC application (Table 1). Further, EPTC differentially inhibited the alkane, ketone, and secondary alcohol fractions, which are primarily C_{29} in chain length leading to a decrease in nonocosane, and its oxidation products. These observations support Kolattukudy's hypothesis that thiocarbamates inhibit the elongation of fatty acids which are decarboxylated to form the C_{29} components of *Brassica* wax (17). Using slices of pea leaves Kolattukudy and Brown (17) showed a concn-dependent inhibition of wax constituents in pea by thiocarbamates. Synthesis of alkanes, secondary alcohols, and ketones was most affected, followed by primary alcohols, aldehydes, and long chain esters. Interestingly low concn (3μM) of CDEC (α-chloroallyl diethyldithiocarbamate) stimulated wax ester synthesis.

The chemical composition of cuticular wax extracted from EPTC-treated cabbage was different from that of the control, however, the alteration in chemistry was not identical to that observed for the epicuticular wax fraction (Table 1, 3). When expressed as a percentage of total cuticular wax, all chemical constituents except fatty acids (+36.9%) and the unidentified polar fraction (+14.6%) were reduced. There was no clear differential inhibition of the alkane, ketone, and secondary alcohol fractions, or stimulation of ester synthesis, as observed in the epicuticular wax fraction. Chemical classes containing long chain compounds (alkanes, ketones, esters, aldehydes, secondary alcohols, and primary alcohols) were reduced by 51.6% when compared to the control (Table 3).

Generally the distinction between epicuticular and cuticular wax is based on the location and the method utilized to extract wax from the cuticular membrane. Often no differentiation is made between the two. Refluxing isolated cuticles with chloroform or chloroform:methanol (1:1) is usually employed to remove cuticular wax embedded within the cutin matrix (1, 9). We observed that cuticular wax contained higher percentages of polar compounds (fatty acids and primary alcohols) than epicuticular wax (Table 1, 3) which is consistent with the observations of Baker and Bukovac (1), who investigated the epicuticular and cuticular wax chemistry of several weed species. Such data do not provide conclusive evidence that epicuticular and cuticular waxes are necessarily different from polar compounds found in the cuticular wax fraction, but may reflect the method of extraction, or a nonuniform distribution of wax constituents within the cuticular membrane.

The differential effect of EPTC on the alteration of cuticular wax in comparison with epicuticular wax is not inconsistent with the suggestion that fatty acid elongation is inhibited. Although, alkane, ketone, and secondary alcohol fractions were not selectively reduced in cuticular wax as in epicuticular wax, their quantity was lower than in the control (Table 1, 3). The marked reduction of all constituents, except the fatty acids and unidentified polar compounds in cuticular wax may have resulted from greater solvent penetration during eipcuticular wax extraction, (prior to isolation of cuticular membrane)

due to the reduced amount of wax present on treated plants. If one can confirm that the biosynthesis and deposition of epicuticular and cuticular waxes are affected similarly by EPTC, then the observed differences are likely a result of differential wax extraction.

Epicuticular wax chemistry may affect wettability and retention of aqueous spray solutions (12), but little direct evidence is available concerning the effect of wax chemistry on cuticular permeability. In experiments using model systems, attempts have been made to assess the influence of wax on passage of water through artificial membranes impregnated with various epicuticular wax components (2, 11). Penetration of water is dependent on the quantity, and the quality of the wax present. When plated in equal amounts esters and alcohols are more permeable than alkanes. This observation would imply that cuticles on EPTC-treated plants would be expected to be more permeable to polar compounds because of a higher content of esters and lower content of alkanes.

It is clear that inhibition of cuticle development results in greater injury following herbicidal sprays (5, 10, 20). Epicuticular wax fine-structure and chemistry influence retention, and cuticular permeability, unfortunately the contribution of each factor to increased efficiency in pesticide application is not completely understood. Cuticles with modified epicuticular wax chemistry may offer a unique test system to further our understanding of the role of wax in relation to retention and penetration of foliar-applied chemicals.

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