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EFFECTS OF ENVIRONMENT ON THE COMPOSITION OF EPICUTICULAR WAX FROM KALE AND SWEDE

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Key Word Index—*Brassica oleracea* var. *acephola*; kale; *B. napus* var. *rapifera*; swede; Cruciferae; effects of environment; leaf epicuticular wax; wax ultrastructure; wax composition.

Abstract—The composition of leaf epicuticular waxes of two genotypes each of kale and swede were determined by gas chromatography–mass spectrometry. Plants were grown indoors (I) and outdoors (O) at SCRI, Scotland, and outdoors at Wädenswil in Switzerland (S). Epicuticular waxes from outdoor-grown plants (O and S) were found to have higher proportions of *n*-alkanes, octacosanoic acid, primary alcohols and long-chain esters but lower proportions of aldehydes, ketones, ketols and secondary alcohols than waxes from (I)-grown plants. Outdoor-grown plants were also found to have proportionally more shorter chain length compounds and indoor-grown plants proportionally more compounds of longer chain length. Variations in wax composition between genotypes of a species and between species were also observed. Differences in leaf surface wax ultrastructure, between species, and between different growth conditions were detected using scanning electron microscopy. The possible role of leaf wax chemicals in the antixenotic resistance to the turnip root fly, *Delia floralis*, of certain genotypes was also considered.

INTRODUCTION

Epicuticular waxes of higher plants provide a physiochemical barrier that aid plants in their resistance to drought and disease [1–3], whilst additionally having a role in host-plant recognition for certain insects [4–7]. The constitution of epicuticular waxes varies widely between species [1] and is also affected by plant age [8–11]. Changes in the environmental conditions to which plants are exposed, particularly heat, humidity and light intensity, can lead to differences in wax morphology [1, 12–16] and composition [1, 14, 17–19]. Of particular interest is the role of epicuticular wax in plant–insect interactions, such as infestation of various plant species by aphids and attack of *Brassica* species by turnip and cabbage root flies. Previous studies have related such interactions to the degree of glaucousness of host plant leaves [4, 5, 20–22] and to the presence or absence of certain wax constituents including alkanes [20, 23], fatty acids [23, 24], ketones, diketones, hydroxy ketones [5, 23], primary alcohols, secondary alcohols, diols [23] and wax esters [7, 25, 26].

Investigation of such interactions requires detailed compositional analysis of all epicuticular wax constituents, including intact wax esters, obtained from resistant and susceptible plant genotypes. We have recently developed a methodology for separation and analysis of epicuticular wax components, including intact wax esters, from brassicas [27]. Herein, we report the results of a compositional analysis of epicuticular wax, including intact wax esters, from swede (*Brassica napus* L. var. *rapifera*) genotypes Doon Major (commerical cultivar) and GRL aga (SCRI breeding line), and kale (*Brassica oleracea* L. var. *acephola*) genotypes Fribor and DGC (dwarf green curly kale) grown under different environmental conditions. Plants were grown indoors (I) and outdoors (O) at SCRI, Dundee and outdoors (S) at Wädenswil in Switzerland. These genotypes have previously been shown to display a range of susceptibility or antixenotic resistance to turnip root fly, *Delia floralis* (Fall.), expressed as non-preference during egg-laying [28].

RESULTS AND DISCUSSION

Wax morphology

The adaxial leaf surface waxes of kale and swede were found to exist in a number of distinct crystalline forms on examination by low-temperature scanning electron microscopy. The predominant forms were tubes, deformed tubes and to a lesser extent rods, generally lying perpendicular to the cuticle surface (Fig. 1). There were also small amounts of dendritic lattice-like crystals protruding from the upper regions of the perpendicular structures, in wax from both species (I); these are parti-

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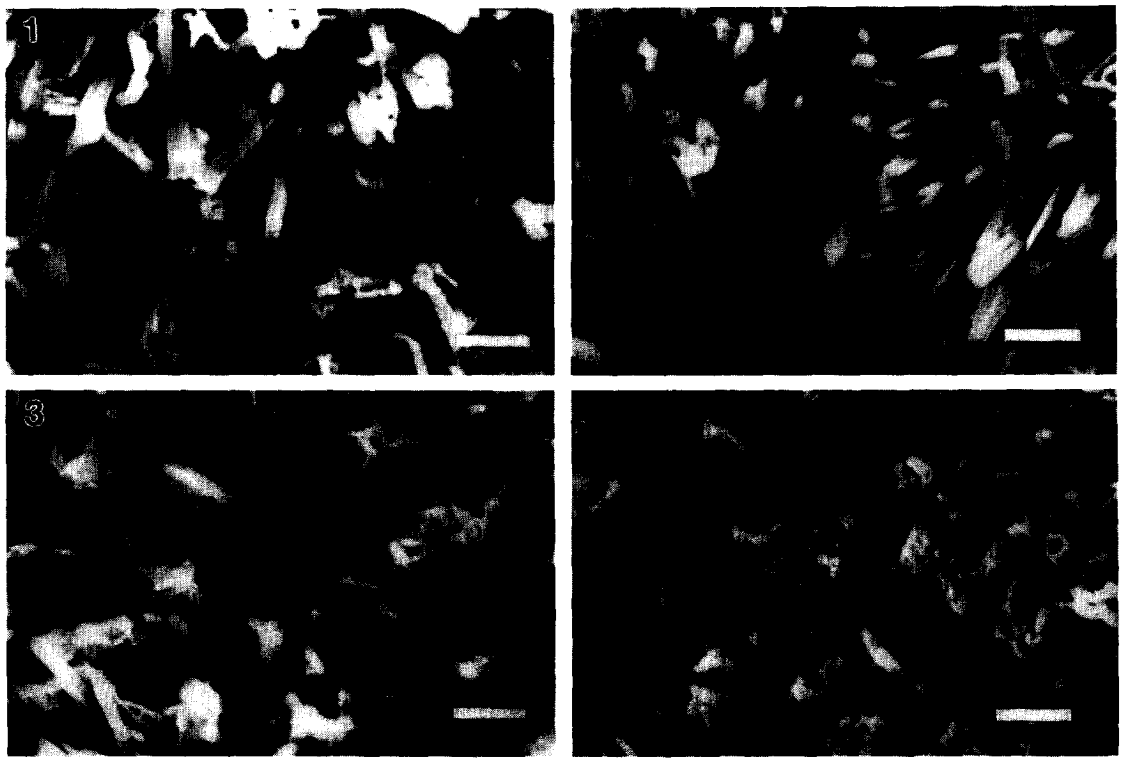

Fig. 1. Scanning electron micrographs of adaxial leaf surfaces of kale genotype, Fribor (F) and swede genotype, Doon Major (DM), grown indoors (I) and outdoors (O) at SCRI. **1**, F(I, ×5000); **2**, F(O, ×5000); **3**, DM(I, ×5000); **4**, DM (O, ×5000). Scale bars represent 2 μm

cularly evident for the swede Doon Major (Fig. 1). The crystalline wax structures appeared to be longer for (I) than for (O) for both species. A number of additional crystalline forms, including plates, needles and more irregular structures are also visible in wax from Doon Major (O Fig. 1). However, these may have been hidden in the electron micrographs of the other samples by the visible structures and may therefore be present in all the samples examined.

Rods, tubes and dendritic lattices are all crystalline forms particularly associated with waxes of rape (*Brassica napus*) and Brussels sprouts (*B. oleracea* var. *gemmifera*) [1, 13–16]. The preponderance of tubes and rods is a characteristic of temperatures around 16° [1, 13–16] and, conversely, dendrites located at the top of supporting structures are characteristic of higher temperatures [13, 14, 16]. The presence of dendrites in wax from kale and swede (I) is indicative of the fluctuation in glasshouse temperatures over the growth period, when temperatures ranged from 20 to 25°.

Wax yields

Average yields of epicuticular wax expressed as both mg kg^{-1} leaf and mg $leaf^{-1}$ are shown in Table 1. Due to the wide variation between the two replicate analyses, it is difficult to draw any firm conclusions concerning quantitative differences in wax yields (O and I). However, yields of wax from Swiss-grown plants (S) were much greater than those from plants grown at SCRI (O, I). This is in agreement with previous studies with various plant species, including those from the Brassicae, showing that there is a concomitant increase in wax yield with increased light intensity [1, 12–19].

Wax composition

Separation of epicuticular wax components from the kale and swede genotypes by capillary gas chromatography following TMSi derivatization gave two well-separated groups of chromatographic peaks. The first group (R_t 23–43 min), with carbon numbers in the range C_{27}–C_{32}, included alkanes, ketones, aldehydes, primary and secondary alcohols, α-hydroxy ketones, β-hydroxy ketones and a fatty acid; these are all well-documented components of *Brassica* epicuticular wax [27, 29–33]. The second group of chromatographic peaks ($R_t > 40$ min) were long-chain wax esters [27]. Individual wax components were identified by combined gas chromatography–mass spectrometry (GC-MS).

The general distribution of individual members of the different chemical classes found in the epicuticular waxes of kales and swedes (Table 2) were similar to those previously reported for Brussels sprouts [14, 29, 30] and rape [33]. A number of these wax components were found to consist of positional isomers, co-eluting on GC. The proportions of individual positional isomers, shown

Table 1. Yields of epicuticular wax from leaves of kale genotypes, Fribor and DGC, and swede genotypes, Doon Major and GRL*

	F†			DGC†			GRL†			DM†		
	I	O	S	I	O	S	I	O	S	I	O	S
Wt of leaves‡	1084	1487.5	2500	1178	1373.5	2500	1424	1497	1909	1786	1542	2537
extracted (g)	±75	±58.5		±41	±115.5		±169	±76		±53	±91	
Wax yield												
mg	511.5	557	5112	287.5	428	3822	374	340.5	1942	351	258.5	2787
	±66.5	±34		±1.5	±66		±37	36.5		±81	±17.5	
mg kg^{-1} leaf	478.4	375.9	2045	244.4	317.9	1529	263.3	229.3	1017	195.4	167.5	1098
	±94.4	±37.6		±9.8	±74.8		±5.3	36.0		±39.5	±1.5	
mg $leaf^{-1}$	3.41	3.71		1.92	2.85		3.12	2.84		2.93	2.15	
	±0.44	±0.23		±0.01	±0.44		±0.31	±0.30		±0.68	±0.15	

*I, grown indoors (glasshouse, SCRI); O, grown outdoors (SCRI); S, grown outdoors (Switzerland).

†Average of two replicate experiments (O and I).

‡A total of 150 leaves from kale genotypes and 120 leaves from swede genotypes were extracted (O and I).

in Table 3 [32, 34], were determined by GC-MS using reconstructed single ion chromatograms (SIC) of major mass spectral fragmentation products. Details for each chemical class are given in the footnotes to Table 3.

The hydrocarbons consisted predominantly of odd carbon *n*-C_{27}, C_{29} and C_{31} compounds, with C_{29} being the major homologue; small amounts of even carbon *n*-C_{28} and C_{30} alkanes were also detected (Table 2). The proportions of C_{27} and C_{29} alkanes were greater in wax from outdoor-grown plants (O, S) than in that from indoor-grown plants (I), whereas the C_{31} alkanes were more abundant in (I) than in (O) or (S). The levels of C_{28} alkanes were greater for (O) than (I), while those of the C_{30} compounds were similar (I, O and S). The C_{27} and C_{28} alkanes were more abundant in wax from kale than swede under all conditions (I, O and S), the C_{28} and C_{30} compounds were of similar abundance, while the C_{31} compounds were more abundant in wax from swede and the kale genotype DGC than in that from the kale genotype, Fribor.

Three coeluting C_{29} secondary alcohols, nonacosan-15-ol (47–73%), nonacosan-14-ol (27–53%) and nonacosan-13-ol were detected as their TMSi derivatives (Table 2), the former being the major isomer while the latter was present in trace amounts only (Table 3). The distribution of these compounds was similar to those found in previous studies of brassica waxes [29, 33, 34]. For all kale and swede genotypes, levels of C_{29} secondary alcohols were greater in (I) than in (S). Their abundances in swede wax were also greater in (I) than in (O), but were similar for kale (I and O). The C_{29} secondary alcohols were more abundant in wax from Fribor than DGC (kale; I and O) and in wax from GRL than Doon Major (swede; I and O) (Table 2). There were no differences in the distribution of positional isomers in wax from the kale Fribor under the different growth conditions, however, relative levels of nonacosan-15-ol were greater in (O and S) than in (I), while those of nonacosan-14-ol were greater in (I) than in (O and S), in wax from the swede Doon Major (Table 3). Relative amounts of nonacosan-15-ol were greater in (O) than in (I and S) for the kale DGC, but were greater in (I and S) than in (O) for the swede GRL, with concomitant changes in the opposite sense for nonacosan-14-ol. Nonacosan-15-ol was relatively more abundant in wax from swede than kale (I and S) and was also more abundant in wax (O) from DGC (kale) and Doon Major (swede) than in wax from the other kale and swede genotypes (Table 3).

Four ketones, all odd-carbon C_{29} compounds, coeluted on capillary GC (Table 2) and were identified as nonacosan-15-one (36–60%), nonacosan-13-one (17–29%), nonacosan-14-one (11–21%) and nonacosan-12-one (11–23%) (Table 3). The former two ketones were the major components of the mixture, with nonacosan-15-one predominating. The distribution of these ketones is different from that reported previously from rape (*B. napus*) and *B. oleracea*, where nonacosan-15-one was the major isomer (94–94%) and nonacosan-14-one a minor constituent (5–6%), with trace amounts nonacosan-13-one (*B. napus*) [29, 33]. The proportions of C_{29} ketones were greater in (I) than in (O) for all genotypes and were also greater in (I) than in (S) for all except the swede GRL. Amounts in (O) were greater than those in (S) for Fribor and Doon Major, but were greater in (S) than in (O) for DGC than in GRL. In general, C_{29} ketones were more abundant in wax from kale than swede (Table 2). There were also fluctuations in the proportions of the four nonacosanone positional isomers (Table 3) but there were no patterns of change common to all genotypes. However, the relative proportions of nonacosan-15-one were generally greater in (O and S) than in (I) whereas those of nonacosan-13-one were greater in (I) than in (O and S), in wax from kale. Changes in the relative proportions of nonacosan-15-one and nonacosan-13-one in wax from the swede Doon Major were in the opposite direction from those seen for kale. Levels of nonacosan-15-one were considerably higher relative to those of the other positional isomers in (I), while those of nonacosan-14-one in (I), nonacosan-13-one in (I) and nonacosan-12-one in (I and S) were lower, in wax from the swede Doon

Table 2. Chemical composition of epicuticular wax from leaves of kale genotypes, Fribor and DGC, and swede genotypes, Doon Major and GRL*

	Fribor†			DGC†			GRL†			DoonMajor†		
	I	O	S	I	O	S	I	O	S	I	O	S
Main group	87.70	79.73	85.94	85.74	81.79	87.64	85.57	80.73	85.18	83.11	74.08	85.49
(*n*-acyl derived)	±1.12	±0.15		±0.12	±1.50		±1.72	±2.20		±0.68	±1.62	
Primary alcohols	1.07	1.35	1.08	1.16	1.56	2.24	0.52	1.04	1.18	0.59	1.43	0.89
	±0.05	±0.05		±0.27	±0.42		±0.02	±0.01		±0.00	±0.08	
Esters	8.79	16.07	9.09	11.32	14.62	8.56	11.81	16.24	11.10	13.94	22.73	11.28
	±0.29	±0.59		±0.50	±0.89		±1.97	1.86		±0.75	±1.26	
Components of main group (normalized, excluding esters and primary alcohols)												
n-alkanes												
C_{27}	0.47	0.70	0.66	0.52	0.71	0.71	0.18	0.38	0.31	0.19	0.36	0.30
	±0.01	±0.02		±0.01	±0.02		±0.04	±0.08		±0.01	±0.00	
C_{28}	0.30	0.46	0.35	0.32	0.40	0.44	0.17	0.22	0.19	0.13	0.21	0.16
	±0.02	±0.00		±0.01	±0.01		±0.02	±0.02		±0.00	±0.01	
C_{29}	46.05	47.95	52.54	44.12	48.80	51.65	42.46	48.64	51.17	42.36	48.84	51.92
	±1.58	±1.59		±2.15	±0.21		±4.48	±2.53		±2.10	±0.73	
C_{30}	0.60	0.55	0.55	0.65	0.54	0.46	0.61	0.58	0.56	0.67	0.58	0.56
	±0.05	±0.04		±0.12	±0.02		±0.06	±0.02		±0.06	±0.06	
C_{31}‡	1.72	1.64	1.31	5.26	4.61	3.51	7.60	6.18	3.98	4.88	4.62	3.99
C_{29} Ketones§	31.82	29.84	26.59	34.79	25.44	30.45	29.45	27.04	27.67	31.33	28.31	25.21
+ C_{31} alkane	±1.46	±4.14		±5.75	±1.46		±5.33	±2.74		±4.17	±3.50	
C_{29} Secondary**,¶	1.17	1.33	0.42	0.71	0.89	0.65	1.17	0.63	0.48	0.75	0.44	0.51
alcohols	±0.00	±0.00		±0.33	±0.13		±0.11	±0.06		±0.03	±0.17	
C_{29} Ketones¶,‖	30.10	28.20	25.28	29.53	20.83	26.94	21.85	20.86	23.69	26.45	23.69	21.22
C_{27} α-Ketols¶	0.22	0.16	0.18	0.28	0.13	0.27	0.16	0.16	0.36	0.21	0.33	0.37
	±0.06	±0.04		±0.10	±0.00		±0.03	±0.03		±0.00	±0.09	
C_{29} α-Ketols¶	1.22	0.89	0.26	1.26	0.67	0.85	1.12	0.57	0.64	1.20	0.56	0.63
	±0.04	±0.12		±0.00	±0.17		±0.12	±0.05		±0.04	±0.25	
C_{29} β-Ketols¶	0.24	0.12	0.07	0.35	0.11	0.17	0.25	0.69	0.08	0.14	0.11	0.06
	±0.12	±0.04		±0.25	±0.00		±0.07	±0.61		±0.06	±0.01	
Aldehydes												
C_{28}††‡‡	0.10	0.08	tr	0.04	0.03	0.03	0.20	tr	0.01	tr	tr	tr
C_{30}	4.13	3.19	1.46	2.34	1.84	1.35	10.73	3.13	3.06	6.44	2.13	2.77
	±0.01	±0.08		±0.88	±0.36		±0.37	±0.13		±0.57	±0.92	
C_{32}	0.22	0.24	0.08	0.10	0.07	0.00	0.47	0.17	0.15	0.21	0.14	0.12
	±0.02	±0.01		±0.02	±0.01		±0.04	±0.01		±0.03	±0.02	
C_{28} acid	9.70	9.57	11.38	11.23	16.18	8.85	10.25	14.16	11.14	12.94	13.86	13.74
	±0.91	±2.14		±5.46	±0.53		±0.38	±0.19		±1.59	±4.52	
Primary alcohols (n-*compounds, excluding* br-*compounds*)‡‡												
C_{26}	0.24	0.38	0.30	0.29	0.46	0.58	0.08	0.19	0.20	0.10	0.27	0.14
	±0.02	±0.04		±0.08	±0.17		±0.01	±0.00		±0.01	±0.03	
C_{28}	0.15	0.17	0.19	0.20	0.19	0.25	0.11	0.22	0.21	0.10	0.25	0.15
	±0.01	±0.02		±0.02	±0.05		±0.00	±0.03		±0.00	±0.01	
C_{30}	0.03	0.02	0.03	0.03	0.01	0.02	0.02	0.02	0.03	0.01	0.01	0.01

*I, grown indoors (glasshouse, SCRI); O, grown outdoors (SCRI); S, grown outdoors (Switzerland).

†Results of two replicates (I and O), percentage composition values given are based on capillary GC peak areas; compounds were identified by GC-MS.

‡Values derived from integration of reconstructed single ion chromatograms (SIC) for $[M]^+$ by comparison of C_{29} ($m/z = 408$) with C_{31} ($m/z = 436$).

§These compounds could not be separated by GC.

‖Calculated by subtraction of values for the C_{31} alkane.

¶These compounds contain positional isomers which co-elute on GC, their distribution, derived from GC-MS, is shown in Table 3.

**Includes C_{28} aldehyde.

††Coelutes with C_{29} secondary alcohols, values determined from integration of reconstructed single ion chromatograms (SIC) for $[M]^+$ by composition of C_{28} ($m/z = 408$) with C_{30} ($m/z = 436$).

‡‡Identified as TMSi ethers [27].

Table 3. Distribution of positional isomers in secondary alcohols, ketones, α-hydroxyketones and β-hydroxyketones in epicuticular wax of leaves of kale genotypes, Fribor and DGC, and swede genotypes, Doon Major and GRL*

	Fribor			DGC			GRL			Doon Major		
	I	O	S	I	O	S	I	O	S	I	O	S
C_{29} Secondary alcohols†												
Nonacosan-15-ol	49	50	51	51	72	47	65	47	72	61	71	63
Nonacosan-14-ol	51	50	49	49	28	53	35	53	28	39	29	27
C_{29} Ketones‡												
Nonacosan-15-one	40	43	46	37	42	42	36	38	42	60	40	51
Nonacosan-14-one	15	15	12	15	15	13	21	20	13	11	10	13
Nonacosan-13-one	29	25	22	28	22	28	24	21	26	17	27	25
Nonacosan-12-one	16	17	20	20	21	17	19	22	19	12	23	11
C_{27} α-hydroxyketones§												
13-Hydroxyheptacosan-12-one	50	47	47	47	48	49	48	46	47	40	43	37
14-Hydroxyheptacosan-13-one	50	53	53	53	52	51	52	54	53	60	57	63
C_{29} α-hydroxyketones§												
14-Hydroxynonacosan-15-one	55	58	63	54	54	55	51	59	62	62	60	69
15-Hydroxynonacosan-14-one	45	42	37	46	46	45	49	41	38	38	40	31
C_{29} β-hydroxyketones ‖												
13-Hydroxynonacosan-15-one	35	43	53	42	46	46	42	46	47	45	44	49
14-Hydroxynonacosan-16-one	65	57	47	58	53	54	58	54	53	55	56	51

*I, grown indoors (glasshouse, SCRI); O, grown outdoors (SCRI); S, grown outdoors (Switzerland).

†Determined from the areas of reconstructed single ion chromatograms (SIC) of mass spectral fragmentation products following cleavage α to the OTMSi ether group of TMSi derivatives [34] (nonacosan-15-ol: m/z = 299; nonacosan-14-ol: m/z = 313, 285).

‡Determined from the SIC of fragmentation products following cleavage α to the carbonyl group (nonacosan-15-one: m/z = 225; nonacosan-14-one: m/z = 221, 239; nonacosan-13-one: m/z = 197, 253; nonacosan-12-one: m/z = 183, 267).

§Determined from intensity of fragmentation products following cleavage α to the OTMSi ether and carbonyl groups of TMSi derivatives [32], measured at the apex of the GC-MS total ion chromatogram (TIC) peaks (13-hydroxyheptacosan-12-one: m/z = 183, 299; 14-hydroxyheptacosan-13-one: m/z = 197, 285; 14-hydroxynonacosan-15-one: m/z = 225, 285; 15-hydroxynonacosan-14-one: m/z = 211, 299).

‖Determined from intensity of fragmentation products following cleavage α to the OTMSi ether and both α and β to the carbonyl groups of TMSi derivatives [32], measured at the apex of the TIC peaks (13-hydroxynonacosan-15-one: m/z = 225, 233, 238, 251, 271, 313, 328, 341; 14-hydroxynonacosan-16-one: m/z = 211, 237, 252, 285, 327, 342).

Major, than in wax from the other kale and swede genotypes (Table 3).

The identified hydroxyketones (as TMSi derivatives) were predominantly C_{29} compounds, the coeluting α-ketols 14-hydroxynonacosan-15-one (51–69%) and 15-hydroxynonacosan-14-one (31–49%) (Tables 2 and 3) and the coeluting β-ketols, 14-hydroxynonacosan-16-one (47–65%) and 13-hydroxynonacosan-15-one (35–53%) (Tables 2 and 3). Small quantities of the C_{27} α-ketols, 13-hydroxyheptacosan-12-one (43–50%) and 14-hydroxyheptacosan-13-one (50–60%) were also detected (Tables 2 and 3). The overall distribution of the C_{29} compounds, with 14-hydroxynonacosan-15-one and 14-hydroxynonacosan-15-one being the predominant isomers, was similar to those reported from earlier studies of the ketol content of brassica epicuticular wax [32]. To our knowledge, C_{27} α-hydroxyketones have not previously been identified in brassica epicuticular wax. The distributions of these ketols were similar to those of the analogous C_{29} compounds, the C_{27} ketols being shorter by a C_2 unit on the ketone side of the molecule. Levels of C_{27} α-ketols in epicuticular wax were greater in (S) than in (I) for the swede genotypes, and were greater in (S) than in (O) for DGC (kale) and GRL (swede) (Table 2). In contrast, amounts of C_{29} α- and β-ketols were greater in (I) than in (O and S) for all genotypes, except for the swede GRL, where the β-ketol was more abundant in (O) (Table 2). There were no changes in the distribution of positional isomers of the C_{27} α-ketols under the different growth conditions (Table 3). However, 14-hydroxynonacosan-15-one was in general relatively more abundant in wax from (S and O) than in (I) (Table 3). For the β-ketols, the relative proportions of 13-hydroxynonacosan-15-one were slightly greater in (O and S) than in (I) (Table 3). 14-Hydroxynonacosan-15-one was relatively more abundant and 15-hydroxynonacosan-14-one less abundant in wax from the swede Doon Major in (I) than from the other kale and swede genotypes (Table 3). 14-Hydroxynonacosan-16-one was relatively more abundant and 13-hydroxynonacosan-15-one less abundant in (I) from wax obtained from the kale Fribor than from the other genotypes (Table 3).

Three aldehydes, n-C_{28} and C_{30} and C_{32} were identified, the C_{30} compound being the major homologue. Trace amounts of an odd carbon number C_{29} aldehyde were also detected. All aldehydes were more abundant in wax from (I) than in (O and S). The proportions of the C_{30} and C_{32} compounds were greater in wax from swede than kale (I and S) (Table 2).

The only free acid detected was the n-C_{28} compound. A number of branched and unbranched acids, including n-C_{28}, were present in wax esters. In general, the proportions of the C_{28} free acid were greater in epicuticular wax from (O and S) than (I). The acid was less abundant in wax from the kales Fribor (O) and DGC (S) than in wax from the other genotypes (Table 2).

More than 50 wax esters in the range C_{42}-C_{49} were detected, which consisted of n- and iso-(i-) C_{16}, C_{18}, C_{20} and $anteiso$-(a-) C_{17} and C_{19} acids esterified to n- and i-C_{26}, C_{28}, C_{30} and a-C_{27} and C_{29} alcohols. The same primary alcohols found in wax esters were also detected in the free form within the epicuticular wax. The proportion of total esters in epicuticular wax was found to be greater in (O) than in (I) for all four *Brassica* genotypes (Table 2). Full details of the composition of the wax esters and primary alcohols will be presented elsewhere.

The proportions of the C_{26} and C_{28} free primary alcohols were greater in (O and S) than in (I) for all genotypes. There were also more C_{26} (I and O) and C_{28} (I) free alcohols in wax from kale than swede (Table 2).

A generalised representation of the biosynthetic relationships between the chemical components identified in *Brassica* epicuticular wax is shown in Fig. 2 [35–37]. The initial stage of their formation is the synthesis *de novo* of long acyl chains of up to C_{16} and C_{18} by sequential elongation of a C_2 starter unit (usually acetate) with C_2 units (from malonate). Subsequently, the acyl chain-length is further extended from C_{16} to C_{30} or higher by a second elongation system, also using malonate as a C_2 source. The acyl CoA esters formed then give rise to fatty acids (Fig. 2(a)), to alkanes following decarboxylation (Fig. 2(b)), to secondary alcohols, ketones and various polyoxygenated compounds following oxidation/hydroxylation and decarboxylation (Fig. 2(c)) and to aldehydes and primary alcohols on reduction (Fig. 2(d)). Wax esters are synthesized either by direct esterification of fatty acids with fatty alcohols, direct transfer of acyl groups to fatty alcohols or acyl transfer from acyl CoA to fatty alcohols (Fig. 2(e)) [35]. An analogous pathway, beginning with a C_3 starter unit (usually propionate) gives rise to some minor epicuticular wax components, with carbon numbers intermediate between those of the major components. Studies with maize and barley have indicated that there are at least two independent elongation/decarboxylation/oxidation/reduction (EDOR) sys-

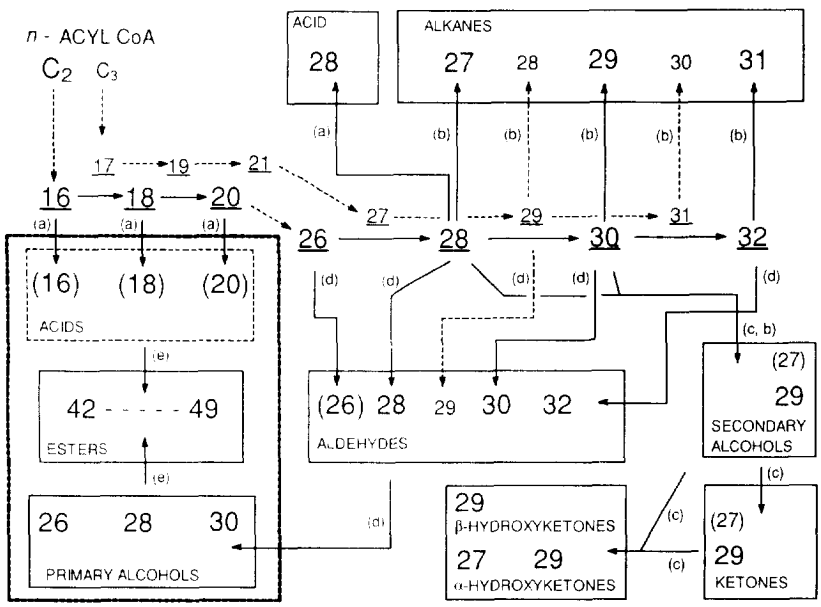

Fig. 2. Schematic representation of biosynthetic routes to compounds of the n-series identified as components in epicuticular wax from the kale genotypes, Fribor and DGC, and the swede, genotypes GRL and Doon Major. Numbers shown are the carbon number of chain-elognation acyl CoA ester intermediates (underlined) and subsequent products derived by (a) release of acid, (b) decarboxylation, (c) oxidation/hydroxylation, (d) reduction, and (e) esterification. Synthesis of esters (outlined) may derive from a different pool of precursors, produced by an analogous pathway. Solid arrows represent major pathways involving even carbon-number n-acyl CoA esters. Dotted arrows represent minor pathways involving odd carbon-number n-acyl CoA esters. Numbers in brackets represent intermediates and products not identified in epicuticular wax, but inferred by subsequent reaction to give identified products.

tems, the first responsible for production of long-chain alkanes, alcohols and aldehydes, the second responsible for synthesis of esters [10, 38, 39].

In brassicas, branched-chain compounds (*br*-) found in wax esters and free primary alcohols appear to originate from use of *br*-precursors, derived from amino acids, in the synthesis *de novo*. A C_4 starter unit (from valine) replacing C_2 in Fig. 2 and a C_5 starter unit (from *iso*leucine) replacing C_3 in Fig. 2, give rise to even carbon-number *i*- and odd carbon-number *a*-compounds, respectively. Branched-chain compounds are not usually found in *Brassica* wax components, such as very long-chain acids, alkanes, aldehydes and ketones etc., but are evidently restricted to long-chain alcohols and esters [27, 29, 31, 40, 41]. Because of the absence of free acids and the presence of free alcohols (*n*- and *br*-) with chain-lengths corresponding to those found in wax esters, it is probable that there are at least two different EDOR systems active in brassicas, as in maize and barley. There may also be two different EDOR systems active in the production of wax ester precursors, for *br*- and *n*- compounds, respectively.

Consequently, when the effects of plant growth under different environmental conditions on the composition of the epicuticular wax from the four *Brassica* genotypes were considered, the wax components could be subdivided into three groups. These were the wax esters, free primary alcohols and the remaining main group of wax components including *n*-alkanes, aldehydes, acids, secondary alcohols and ketols. Further analysis of the effects of environment are here restricted primarily to the latter group of compounds.

Two general effects, apparently common to all four *Brassica* genotypes, were evident. These are clearly shown in Table 4, where the total proportions in wax of the products of reductive reactions (aldehydes) and oxidative reactions (secondary alcohols, ketones and ketols) were less in (O and S) than in (I) while the total proportions of alkanes and the C_{28} acid were greater in (O and S) than in (I). Also, when the total proportions of compounds derived from acyl CoA esters of chain-length 28–32 were compared, it was evident that those with shorter chain-length (28, 29) were relatively more abundant in (O and S) than in (I), while those of longer chain-lengths (31, 32) were more abundant in (I) than in (O and S), with the transition at 30.

There is limited literature regarding changes in wax composition under different environmental conditions. Previous studies with *B. oleracea* suggested that such changes were only evident when extremes of growth conditions were compared [14]. Light stimulates conversion of elongation products to hydrocarbons, secondary alcohols and ketones [14, 17], and this is in agreement with our results when the increase in wax yields on comparison of the extremes of environmental conditions (S with O and I) are taken into account. However, the chain-length distribution of *Brassica* wax components were supposedly unaffected by variation in light levels [17], whereas a 20° temperature increase reduced the levels of the main elongation products by 40% [14]. Elevated temperatures have also been observed to stimulate conversion of aldehydes to primary alcohols in leaves of *Hedera helix* [19]. The chain-lengths of barley (*Hordeum*) epicuticular wax components have been reported to change with variation in light intensity, although the proportions of constituents produced within the EDOR pathways were unaltered [18]. The effects of variation in environmental conditions observed for the kale and swede genotypes in our investigation show some of these features. However, the overall effects are most probably due to the synergism of diurnal variation in local light and temperature levels at the different geographic locations used for these studies.

Our results suggest that the specificity and/or activity of some, or all, of the enzymes involved in the pathways shown in Fig. 2 change under different environmental conditions. A change in chain-length specificity for some enzyme systems is clearly indicated. This may have been active at the chain-elongation stage only, or may have applied more generally. The observed shift from redox products to alkanes and acids on comparison of (O and S) with (I) could arise from a relative increase in decarboxylation, a decrease in redox activity or a combination of both. The reduction in aldehyde levels was general across all chain-lengths, while α-ketol synthesis was greater for C_{27} but less for C_{29}, possibly indicating that the oxidation/hydroxylation system is chain-length sensitive. The reduction in aldehyde levels might be explained by an increase in primary alcohol synthesis and esterification. However, it is generally believed that free primary alcohols and esterified alcohols are produced by a separate elongation/reduction system. Additionally, the proportions of wax esters were only greater in (O), but not in (S), although aldehyde levels were depleted under both growth conditions. Further evidence that changes in enzyme activity may be more general arises from the variation in the distribution of the positional isomers of secondary alcohols, ketones and ketols, which suggests that the specificities of the oxidation/hydroxylation systems also varied under different environmental conditions. The similarity of C_{27} and C_{29} α-ketols on the alcohol side of the molecule, with a C_2 difference on the ketone side of the molecule is in agreement with the view that oxidation occurs during the elongation system prior to decarboxylation, and that the site of oxidation is highly specific.

It is also evident from Tables 2 and 4, that kale epicuticular wax has proportionally more constituents of shorter chain-length than swede wax, again suggesting differences in the specificity of some enzyme systems. These variations in chain-length under the different growth conditions, and between the different *Brassica* species, are also seen for the long-chain wax esters and their component acid and alcohol moieties (details to be presented elsewhere). Since the esters consist primarily of *br*-constituents, which are synthesized by a different EDOR system, the differences in enzyme specificities appear to be general, and suggest a common origin for the various EDOR complexes.

Table 4. Distribution of chemical components, excluding wax esters and free primary alcohols, in epicuticular wax of leaves of kale genotypes, Fribor and DGC, and swede genotypes, Doon Major and GRL, according to biosynthetic origin

	Fribor*			DGC*			GRL*			DoonMajor*		
	I	O	S	I	O	S	I	O	S	I	O	S
Products of redox reactions†	37.40	34.21	27.74	34.61	24.56	30.26	35.95	26.21	28.47	35.40	27.40	25.68
% *change*‡		−8.53	−25.83		−29.04	−12.57		−27.09	−20.81		−22.60	−27.46
C_{28} acid + alkanes§	58.84	60.87	66.79	62.11	71.25	65.61	61.27	70.16	67.35	61.17	68.46	70.67
% *change*‡		+3.45	+13.51		+14.72	+5.64		+14.51	+9.92		+11.92	+15.53
Products derived from acyl (C_n) CoA esters‖												
$n = 28$	0.79	0.94	0.84	0.85	0.87	1.01	0.54	0.55	0.68	0.40	0.68	0.68
% *change*‡		+18.99	+6.33		+2.35	+18.82		+1.85	+25.93		+70.00	+70.00
$n = 29$	0.30	0.46	0.35	0.32	0.40	0.44	0.17	0.22	0.19	0.13	0.21	0.16
% *change*‡		+53.33	+16.67		+25.00	+37.50		+29.41	+11.76		+61.54	+23.08
$n = 30$	82.91	81.67	80.03	78.31	73.13	81.61	77.58	74.52	79.12	77.34	75.77	77.10
% *change*‡		−1.50	−3.47		−6.61	+4.21		−3.94	+1.99		−2.03	−0.31
$n = 31$	0.60	0.55	0.55	0.65	0.54	0.46	0.61	0.58	0.56	0.67	0.58	0.56
% *change*‡		−8.33	−8.33		−16.92	−29.23		−4.92	−8.20		−13.43	−16.42
$n = 32$	1.94	1.88	1.39	5.36	4.68	3.51	8.07	6.35	4.13	5.09	4.76	4.11
% *change*‡		−3.09	−28.35		−12.69	−34.51		−21.31	−48.82		−6.48	−19.25

*Values derived from those for individual chemical components in Table 2.

†Sum of values for compounds derived from acyl CoA esters by oxidation/hydroxylation (secondary alcohols, ketones and ketols; Fig. 2(c)) or reduction (aldehydes; Fig. 2(d)).

‡Values are the ratio: difference between (I) and (O or S)/(I) × 100; a positive sign indicates an increase (O or S) > (I); a negative sign indicates a decrease (I) > (O or S).

§Sum of values for compounds derived from acyl CoA esters directly (C_{28} acid; Fig. 2(a)) or by decarboxylation (alkanes; Fig. 2(b)).

‖Values for n = 28: sum of C_{27} alkanes and a-ketols and C_{28} acids and aldehydes; n = 29: C_{28} alkanes; n = 30: C_{29} alkanes, α- and β-ketols and C_{30} aldehydes; n = 31: C_{30} alkanes; n = 32: C_{31} alkanes and C_{32} aldehydes.

Of the four *Brassica* genotypes used in this investigation, the swede Doon Major is highly susceptible to infestation by the turnip root fly, *Delia floralis* (Fall.), a major pest of brassicas in Northern Europe and Fennoscandia. The other genotypes, GRL, DGC and Fribor, show increasing antixenotic resistance to turnip root fly [28]. Bioassay of leaf surface extracts (prepared from leaves dipped initially in dichloromethane then in methanol) had shown that most of the stimulatory activity was located in the methanol (polar) fractions and was greatest for Doon Major and least for Fribor. However, the dichloromethane (non-polar) extracts showed some activity, particularly from Doon Major. Additionally, polar leaf extracts were only effective in the presence of a hydrocarbon [28, 42]. Behavioural analysis of root fly activity on plants indicated the importance of the leaf surface as the principal stage at which host selection was occurring [28]. It is therefore likely that leaf surface wax components, in addition to specific polar signal compounds, are involved in the overall process of insect host selection.

The distributions of wax yields were very similar to the patterns of antixenotic resistance (Fribor, greatest; Doon Major, least), indicating that wax thickness and/or density may restrict the insects' access to chemical oviposition stimulants. Increased wax levels have been correlated with resistance of cabbage (*B. oleracea*), to the aphid, *Brevicoryne brassicae* L., of sorghum (*Sorghum bicolor*) to the green bug, *Schizaphis graminum* (Rondani), of winter wheat (*Triticum aestivum*) to the English grain aphid, *Sitobion avenae* (F), and variation in the distribution of spotted alfalfa aphids, *Therioaphis maculata* (Buckton), in the foliar canopy of alfalfa (*Medicago sativa*) [5, 21, 22, 43, 44].

Specific wax components may also be involved in the plant/insect interaction. Alkanes promote movement of pea aphids, *Acrythosiphon pisum* (Harris), to feeding sites on the abaxial surfaces of *Vicia faba* leaves [20] and they have been identified as possible feeding and oviposition stimulants in other studies [23]. The settling of alate green peach aphids, *Myzus persicae* (Sulzer), on hosts was deterred by short-chain fatty acids (C_3-C_{13}) but was stimulated by fatty acids of chain-length $> C_{16}$ [24]. Various carboxylic acids, including palmitic acid (n-C_{16}), applied to leaves of cauliflower (*B. oleracea* var. *gemmifera*), also deterred oviposition by the cabbage root fly, *Delia radicum* (L.) [45]. Antixenotic resistance of alfalfa to *T. maculata* has been linked with the abundance of cuticular wax esters; some resistant plants had up to 50% more wax esters than those susceptible to aphid attack [7, 26]. Wax esters deterred feeding by *Locusta migratoria* (L.) on sorghum [25, 46]. Alkanes are unlikely to be directly involved in the resistance/susceptibility patterns of the *Brassica* genotypes studied here, because taking wax yields into account, there were more cuticular hydrocarbons on resistant Fribor than susceptible Doon Major. However, levels of both the C_{28} fatty acid and wax esters were more than 50% greater for Fribor than Doon Major and their distributions in wax of the four genotypes were similar to the patterns of antixenotic resistance. Therefore, these chemicals may have some involvement in conferring antixenotic resistance to *Delia floralis* during host selection for oviposition sites.

Plant resistance to certain insects, including various aphids and neonate diamondback moth (*Plutella xylostella* (L.)) larvae has also been related to the loss of the normal glaucous characteristics of plant leaves in some varieties of wheat, sorghum, kale and cabbage [22–24, 47, 48]. However, none of the *Brassica* genotypes used in this investigation are characterized by loss of glaucousness and the chemical changes associated with this condition are generally opposite to those observed on comparison of resistant and susceptible genotypes. The role of epicuticular wax in the interaction between these *Brassica* genotypes and *D. floralis* requires further investigation, in particular regarding the physical barrier provided by the wax and the activity of specific wax components in their interactions with polar leaf surface oviposition stimuli for *D. floralis*.

EXPERIMENTAL

Plant growth. (*i*) *At SCRI.* Plants were grown (seed sown on 23 June 1993) in 15.2 cm pots containing a Levington^R compost/sand mixt. (3:1) in a glasshouse (I, 20–25°) or outside the glasshouse (O) in a semi-shaded location. Plants were transferred outside at the 1–2 true leaf stage. (*ii*) *At the Swiss Federal Research Station for Fruit-Growing, Viticulture and Horticulture, Wädenswil, Zurich, Switzerland.* Plants were field-grown (S). Seed was sown in a compost/peat mixt. (last week of June 1993) and seedlings were then transplanted outside into the soil (1 July). Plants were harvested at the 10 leaves per plant and 8 leaves per plant stages of growth for kale and swede, respectively (21 September 1993 at SCRI, 4–14 October 1993 in Switzerland). Accumulative solar irradiation levels and total hours of sunshine for the 3 months July–September 1993 were 3.21×10^5 Wh m^{-2} and 456 hr (SCRI) and 3.80×10^5 Wh m^{-2} and 509 hr (Switzerland).

Collection of epicuticular wax. Leaves were detached from (15 plants each at SCRI), of outdoor-grown (O) and indoor (glasshouse)-grown (I) swede genotypes Doon Major (commercial cultivar) and GRL (SCRI breeding line), and kale genotypes Fribor and DGC (dwarf green curly kale). Weights of leaves collected are shown in Table 1. Leaves from each group of plants were dipped in CH_2Cl_2 (500 ml at SCRI, 2 l in Switzerland), the CH_2Cl_2 filtered, evapd to dryness and redissolved in 50 ml of petrol (bp 40–60°). The petrol solns were dried over Na_2SO_4, filtered and evapd to dryness. The resulting solid samples were frozen (− 20°) prior to chemical analysis.

Sample preparation and analysis by GC and GC-MS. Samples were derivatized with *N,O-bis*(trimethylsilyl)-trifluoroacetamide (BSTFA) and analysed by capillary GC and GC-MS at temps of up to 350°, as described previously [27]. Details of instrumentation and chromatographic conditions are also given elsewhere [27].

Scanning electron microscopy. Frozen hydrated leaf samples (5 × 5 mm) were sputter-coated with gold and transferred to the cold stage of the scanning electron microscope for low temp. SEM. Details of instrumentation and procedures are described elsewhere [49, 50].

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