PERGAMON

Phytochemistry 52 (1999) 1239–1254

PHYTOCHEMISTRY

Epicuticular wax composition in relation to aphid infestation and resistance in red raspberry (*Rubus idaeus* L.)

Tom Shepherd*, Graeme W. Robertson, D. Wynne Griffiths, A. Nick E. Birch

Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, UK

Received 30 April 1999; accepted 14 July 1999

Abstract

Epicuticular waxes from the aphid-resistant red raspberry (*Rubus idaeus*) cultivar Autumn Bliss and the aphid-susceptible cultivar Malling Jewel were collected from the newly emerging crown leaves, and also from the group of four more mature leaves immediately below the crown. Resistance and susceptibility status of the leaves to infestation by the large raspberry aphid, *Amphorophora idaei*, were determined by bioassay with the insect just prior to collection of the wax. Analysis showed the waxes to consist of a complex mixture of free fatty acids; free primary alcohols and their acetates; secondary alcohols; ketones; terpenoids including squalene, phytosterols, tocopherol and amyrins; alkanes and long chain alkyl and terpenyl esters. Compositional differences which may relate to *A. idaei*-resistance status were noticeably higher levels of sterols, particularly cycloartenol, together with the presence of branched alkanes, and an absence of C_{29} ketones and the symmetrical C_{29} secondary alcohol in wax from the resistant cultivar Bliss. There were also differences between the cultivars in the distribution of individual amyrins and tocopherols and in the chain length distribution for homologues of fatty acids, primary alcohols and alkanes, and these may also be related to resistance to *A. idaei*. Emerging leaves had lower levels of primary alcohols and terpenes, but higher levels of long-chain alkyl esters, and in general, more compounds of shorter chain-length than the more mature leaves. During bioassay *A. idaei* displayed a preference to settle on the more mature leaves. This may be due to greater wax coverage and higher levels of the compounds of shorter chain length found in the newly emerged younger leaves at the crown of the plant.

Keywords: *Rubus idaeus*; Red raspberry; Rosaceae; Leaf epicuticular wax; Wax composition; *Amphorophora idaei*; Raspberry aphid; Aphidae; Aphid resistance; Aphid susceptibility

1. Introduction

Raspberry and blackberry, members of the *Rubus* genus (family Rosaceae), are important soft fruit crops in temperate climates. Among the insect pests which infest these plants, the large raspberry aphid, *Amphorophora idaei* (Börner), is of particular economic importance being the only vector of significance for several viruses which infect raspberry in Europe (Jones, 1979, 1986). Evidence exists that the leaf surface of *Rubus*, and in particular the cuticular wax, plays a significant and possibly decisive role in determining resistance and susceptibility to infestation by the insect (Robertson, Griffiths, Birch, Jones, McNicol & Hall, 1991) and has been suggested for several other plant species subject to aphid attack (Klingauf, Nöcker-Wenzel & Röttger, 1978; Phelan & Miller, 1982; Eigenbrode, Espelie & Shelton, 1991).

In comparison to other plant species, data on cuticular wax composition for members of the Rosaceae is limited to only a few examples from the leaves of peach, *Prunus persica* L. (Baker, Bukovac & Flore, 1979; Bukovac, Flore & Baker, 1979) and strawberry, *Fragaria* spp. (Baker & Hunt, 1979), the fruit epidermis of plum, *Prunus domestica* L. (Ismail, Brown, Tucknott, Holloway & Williams, 1977), and the flowers of decorative roses, *Rosa* spp. (Mladenova,

* Corresponding author. Tel.: +44-1382-562731; fax: +44-1382-562426.

E-mail address: tsheph@scri.sari.ac.uk (T. Shepherd).

0031-9422/99/$ - see front matter
PII: S0031-9422(99)00413-6

Table 1
Yields of epicuticular wax from leaves of raspberry cultivars, Autumn Bliss and Malling Jewel, and bioassay results for resistance to *A. idaei*

	Autumn Bliss[a] (resistant)						Malling Jewel[a] (susceptible)					
	Open to aphid infestation[b,c]					Bliss[d] tops	Open to aphid infestation[b,c]					Jewel[d] tops
	1	2	3	4	Mean (sd)		1	2	3	4	Mean (sd)	
No of leaves	20	20	20	20			16	16	20	20		
Leaf weight (g)	28.17	33.23	30.98	27.56	29.99 (2.63)	4.85	21.33	25.86	31.36	30.76	27.33 (4.70)	4.90
Wax yield (mg)	32.00	34.50	24.30	30.00	30.20 (4.34)	10.2	20.50	20.70	25.00	32.50	24.68 (5.61)	8.10
Wax yield (mg g^{-1} leaf wt)	1.14	1.04	0.78	1.09	1.01 (0.16)	2.10	0.96	0.80	0.80	1.06	0.90 (0.13)	1.65
Aphids present[e]	0	1	0	0	0.25		207	261	329	401	299.5 (84)	
Additional aphids[f]	0	1	0	0	0.25		5	0	6	9	5.0 (3.7)	
Aphids g^{-1} leaf weight	0	0.03	0	0	0.10		9.70	10.09	10.49	13.04	10.83 (1.50)	
	Aphid-free controls,[b,g]						Aphid-free controls,[b,g]					
	1	2	3	4	Mean		1	2	3	4	Mean	
No. of leaves	20	20	20	20			20	20	20	20		
Leaf weight (g)	34.78	38.04	8.36	18.49	24.92 (13.96)		39.32	39.76	14.74	30.45	31.07 (11.70)	
Wax yield (mg)	42	48.5	7.2	17	28.68 (19.73)		39.7	43	12.2	27.1	30.50 (13.99)	
Wax yield (mg g^{-1} leaf wt)	1.21	1.27	0.86	0.92	1.07 (0.21)		1.01	1.08	0.83	0.89	0.95 (0.11)	
Aphids present[e]	0	0	0	0	0		0	0	0	0	0	
Additional aphids[f]	0	0	0	0	0		0	0	0	0	0	

[a] Samples were taken from the crown of emerging leaves (tops) and from groups of four leaves immediately below the crown.
[b] Values are for individual replicates, means and standard deviations for four replicates each of four or five plants.
[c] Plants subject to bioassay with *A. idaei*.
[d] Combined sample from all plants.
[e] Total numbers of *A. idaei* on the groups of four leaves immediately below the crown.
[f] Total numbers of *A. idaei* on other plant parts.
[g] Control plants, not subject to bioassay with *A. idaei*.

Stoianova-Ivanova & Daskalov, 1976; Mladenova, Stoianova-Ivanova & Camaggi, 1977). We have previously reported a partial analysis of raspberry leaf wax (Robertson et al., 1991), and more recently conducted a detailed analysis of the effects of plant developmental stage and growth conditions on raspberry epicuticular wax, the results of which will be presented elsewhere.

Here, we describe differences in the composition of wax from the *A. idaei*-resistant cultivar, Autumn Bliss, containing the major resistance gene A_{10} (Birch & Jones, 1988) and wax from the *A. idaei*-susceptible cultivar, Malling Jewel, in a continuing study of the ecological role of epicuticular wax in plant leaves (Shepherd, Robertson, Griffiths, Birch & Duncan, 1995b; Shepherd, Robertson, Griffiths & Birch, 1997). An important part of the experiment was to verify the biological activity of the leaf surface by bioassay with *A. idaei* immediately prior to collection and chemical analysis of the epicuticular wax. In this way, a close correlation between observed aphid behaviour and the chemical status of the leaf could be achieved. In addition, a similar aphid-free control group of plants from both cultivars, not subject to bioassay, were also sampled to determine whether the presence of aphids had any induced effect on wax composition.

2. Results and discussion

2.1. Wax yields

Yields of epicuticular wax recovered from leaves of Autumn Bliss and Malling Jewel, along with the results of plant bioassay with *A. idaei*, are shown in Table 1. Four replicate measurements of wax yield and aphid numbers present on older leaves were made for each genotype with or without aphid bioassay. Wax yields for young/newly emerging leaves were made from bulked samples from both genotypes. Wax recoveries from Bliss and Jewel, expressed as a fraction of fresh leaf weight, were very similar under all conditions, and yields were up to two times greater from emerging leaves than the more mature fully expanded leaves. Leaf areas were (visually) similar for both genotypes, and therefore, wax coverage per unit area was similar on both genotypes. Bioassay demonstrated high populations of aphids on Jewel, and virtually

Table 2
General distribution of the different classes of compound in epicuticular wax from raspberry cultivars, Autumn Bliss and Malling Jewel

	Autumn Bliss[a]			Malling Jewel[a]		
	Aphid-free[b,c]	+Aphids[b,d]	Tops[e]	Aphid-free[b,c]	+ Aphids[b,d]	Tops[e]
Acids	2.75	2.88	1.85	2.71	2.57	3.48
	0.20	*0.16*		*0.15*	*0.06*	
Primary alcohols	26.65	27.02	11.78	26.32	25.80	12.29
	1.66	*2.54*		*3.50*	*2.35*	
Alkanes and alkenes	4.86	6.25	5.85	3.42	3.41	8.99
	1.52	*0.82*		*0.20*	*0.61*	
Secondary alcohols	0.18	0.19	n.d.[f]	0.12	0.10	0.14
	0.04	*0.01*		*0.02*	*0.01*	
Ketones	n.d.	n.d.	0.09	0.09	0.09	0.13
				0.01	*0.02*	
Tocopherols	2.12	1.14	0.77	1.66	1.41	0.37
	0.16	*0.23*		*0.25*	*0.28*	
Amyrins	0.55	1.11	0.25	0.45	0.84	0.14
	0.05	*0.45*		*0.13*	*0.11*	
Sterols	3.64	5.62	0.27	0.06	0.10	0.07
	0.18	*1.13*		*0.01*	*0.01*	
Squalene	0.05	0.02	0.08	0.05	0.07	0.04
	0.03	*0.01*		*0.03*	*0.03*	
Alkyl esters	58.05	54.18	78.22	64.20	64.52	73.40
	0.28	*5.13*		*3.14*	*3.11*	
Terpenyl esters	1.14	1.57	0.82	0.89	1.08	0.95
	0.34	*0.38*		*0.31*	*0.17*	
Triacylglycerols[g]	n.d.	n.d.	n.d.	n.d.	0.85	0.52

[a] Samples were taken from the crown of emerging leaves (tops) and from groups of four leaves immediately below the crown. Values are expressed as a percentage of total wax content, excluding triacylglycerols.
[b] Values are means and standard deviations for three replicates each of four or five plants.
[c] Control plants, not subject to bioassay with *A. idaei*.
[d] Plants subject to bioassay with *A. idaei*.
[e] Combined sample from all plants.
[f] n.d.: Not detected.
[g] Values are expressed relative to the total for all other wax components.

none on Bliss, confirming that at the time of sampling, Bliss was aphid-resistant and Jewel was aphid-susceptible.

2.2. Wax composition

The wax was characterised by capillary gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS) following formation of trimethylsilyl derivatives and was found to contain a broad spectrum of components. The overall distribution of these wax constituents which include aliphatic hydrocarbons, fatty acids, primary and secondary alcohols, ketones, and wax esters together with various terpenoids including squalene, sterols, tocopherols and amyrins is shown in Table 2. The distributions of individual compounds within each class are shown in full in Tables 3–6. The overall distribution of the major and minor classes of chemical components found in wax from the more mature biologically active leaves of Bliss and Jewel exposed to *A. idaei* are also shown in diagrammatic form in Fig. 1(a) and (b). Similarly, data for individual components within each class are shown for secondary alcohols (Fig. 1(c)), terpenes, including sterols, amyrins and tocopherols (Fig. 1(d)–(g)), and for the main free fatty acids, primary alcohols and alkanes (Fig. 2(a)–(c)), respectively. Data for older leaves are averages for replicates 1–3 and for emerging leaves data are for bulk samples. The major wax components in all samples were primary alcohols and long-chain alkyl esters.

2.2.1. Fatty acids

Individual free fatty acids (as TMSi esters) were found in the range C_{12}–C_{32}, with even carbon numbers predominant (Table 3, Fig. 2(a)), and of these the C_{22}–C_{30} homologues were most abundant. Two distinct maxima were observed in the acid chain length distribution, at C_{16} and C_{24}, respectively. The first maxima probably reflects the utilisation of acyl chains up to C_{16}–C_{18} as produced initially by the synthesis de novo, whereas the second maxima reflects the utilisation of acyl chains produced by subsequent elongation. Although mainly straight-chain (*n*-) satu-

Table 3
Distribution of free fatty acids in epicuticular wax from raspberry cultivars, Autumn Bliss and Malling Jewel

C_n	ECL[b]	Autumn Bliss[a]			Malling Jewel[a]		
		Aphid-free[c,d]	+Aphids[c,e]	Tops[f]	Aphid-free[c,d]	+Aphids[c,e]	Tops[f]
Acids							
12 : 0	*12.00**	0.49	0.37	0.69	0.62	0.63	0.59
		0.18	*0.06*		*0.27*	*0.12*	
13 : 0	*13.00**	0.16	0.11	0.26	0.13	0.16	0.25
		0.08	*0.03*		*0.04*	*0.02*	
i-14 : 0	*13.59*†	0.09	0.07	0.13	0.05	0.06	0.09
		0.05	*0.03*		*0.01*	*0.02*	
14 : 1	*13.69*†	0.08	0.07	0.08	0.07	0.07	0.05
		0.02	*0.01*		*0.03*	*0.01*	
(*n* − 5) 14 : 1	*13.78*†	0.27	0.26	0.60	0.45	0.56	0.43
		0.06	*0.05*		*0.22*	*0.15*	
14 : 0	*14.00**	1.61	1.58	2.84	2.12	2.38	1.99
		0.16	*0.22*		*0.76*	*0.12*	
br-15 : 0	*14.41*†	0.08	0.07	0.14	0.10	0.11	0.10
		0.01	*0.01*		*0.03*	*0.03*	
br-15 : 0	*14.69*†	0.07	0.06	0.14	0.09	0.10	0.12
		0.01	*0.01*		*0.04*	*0.02*	
a-15 : 0	*14.77*†	0.03	0.03	0.06	0.04	0.04	0.06
		0.01	*0.02*		*0.02*	<[g]	
15 : 0	*15.00**	0.59	0.40	1.40	0.71	0.97	1.02
		0.16	*0.06*		*0.27*	*0.27*	
t-16 : 0	*16.62*†	0.01	0.01	0.07	0.03	0.03	0.04
		<	<		*0.01*	<	
(*n* − 7) 16 : 1	*15.73*†	0.75	0.53	1.04	0.97	1.48	0.93
		0.33	*0.14*		*0.30*	*0.26*	
(*n* − 5) 16 : 1	*15.80*†	0.31	0.25	0.62	0.37	0.68	0.47
		0.08	*0.08*		*0.07*	*0.16*	
16 : 0	*16.00**	7.53	5.94	12.47	7.58	9.44	8.00
		2.39	*0.70*		*1.74*	*0.65*	
br-17 : 0	*16.40*†	0.08	0.06	0.14	0.07	0.09	0.08
		0.03	*0.03*		*0.02*	*0.01*	
br-17 : 0	*16.48*†	0.06	0.06	0.13	0.05	0.07	0.08
		0.02	*0.02*		*0.02*	*0.02*	
(*n* − 9) 17 : 1	*16.73*†	0.08	0.08	0.34	0.22	0.23	0.23
		0.03	*0.01*		*0.08*	*0.02*	
a-17 : 0	*16.80*†	0.05	0.04	0.10	0.07	0.07	0.07
		0.02	*0.01*		*0.02*	<	
17 : 0	*17.00**	0.71	0.64	1.11	0.76	0.86	0.93
		0.21	*0.04*		*0.13*	*0.08*	
(*n* − 6) 18 : 2	*17.67*†	0.28	0.31	0.66	0.43	0.55	0.36
		0.06	*0.03*		*0.13*	*0.05*	
(*n* − 9) 18 : 1	*17.74*†	1.60	1.23	2.92	1.72	2.37	1.84
		0.47	*0.23*		*0.66*	*0.11*	
(*n* − 7) 18 : 1	*17.81*†	0.55	0.53	0.99	0.75	0.92	0.78
		0.04	*0.08*		*0.14*	*0.31*	
18 : 0	*18.00**	4.18	4.06	5.29	3.39	3.78	2.85
		1.22	*0.43*		*1.09*	*0.05*	
19 : 0	*19.00**	0.20	0.19	0.24	0.25	0.33	0.20
		0.06	*0.09*		*0.04*	<	
20 : 0	*20.00**	2.66	2.84	3.49	2.20	1.90	1.81
		0.27	*0.03*		*0.12*	*0.11*	
22 : 0		9.30	9.12	13.75	6.63	6.02	6.07
		1.26	*0.51*		*0.44*	*0.20*	
23 : 0		2.24	2.36	2.23	3.03	2.92	2.10
		0.28	*0.06*		*0.45*	*0.40*	
24 : 0		28.07	29.09	33.89	25.87	24.04	24.95
		3.02	*2.02*		*3.63*	*0.96*	

Table 3 (*continued*)

C_n	ECL[b]	Autumn Bliss[a]			Malling Jewel[a]		
		Aphid-free[c,d]	+Aphids[c,e]	Tops[f]	Aphid-free[c,d]	+Aphids[c,e]	Tops[f]
26 : 0		22.67	21.37	10.64	19.46	17.91	19.41
		0.42	*1.16*		*2.07*	*0.94*	
28 : 0		7.92	9.52	1.64	11.15	10.80	14.08
		0.45	*0.97*		*0.45*	*0.80*	
30 : 0		6.16	7.20	1.70	10.18	9.99	9.13
		0.16	*0.32*		*0.80*	*0.63*	
32 : 0		1.08	1.53	0.20	0.38	0.42	0.87
		0.39	*0.36*		*0.05*	*0.02*	

[a] Samples were taken from the crown of emerging leaves (tops) and from groups of four leaves immediately below the crown. Values are expressed as a percentage of total free acids in each extract.

[b] Equivalent Chain Lengths (ECL). Values for *anteiso-* (*a-*), *iso-* (*i-*) and unidentified branched (*br-*) saturated acids and unsaturated acids (†) were derived by interpolation from a graphical plot of log GC retention times for strait-chain *n*-saturated acids against their ECL values (∗). Double bond positions in unsaturated acids are indicated by the $(n-x)$ notation where n is the chain-length of the acid and x is the number of carbon atoms from the double bond to the terminal methyl carbon atom. Specific examples are also given in the the text.

[c] Values are means and standard deviations for three replicates each of four or five plants.

[d] Control plants, not subject to bioassay with *A. idaei*.

[e] Plants subject to bioassay with *A. idaei*.

[f] Combined sample from all plants.

[g] < : Standard deviation < 0.005.

Table 4
Distribution of primary alcohols and alcohol acetates in epicuticular wax from raspberry cultivars, Autumn Bliss and Malling Jewel

C_n	ECL[b]	Autumn Bliss[a]			Malling Jewel[a]		
		Aphid-free[c,d]	+Aphids[c,e]	Tops[f]	Aphid-free[c,d]	+Aphids[c,e]	Tops[f]
Alcohols							
13 : 0	*13.00*∗	0.01	0.01	0.04	0.01	0.01	0.05
		< [g]	<		<	<	
14 : 0	*14.00*∗	0.01	0.01	0.01	0.01	0.01	0.02
		<	<		<	<	
15 : 0	*15.00*∗	0.02	0.02	0.07	0.02	0.02	0.07
		0.01	<		<	<	
br-16 : 0	*15.41*†	0.01	0.01	0.02	0.01	0.01	0.02
		<	<		<	<	
16 : 0	*16.00*∗	0.01	0.02	0.04	0.01	0.02	0.05
		<	<		<	*0.01*	
17 : 0	*17.00*∗	0.01	0.01	0.02	0.01	0.01	0.03
		<	<		<	<	
br-18 : 0	*17.44*†	0.01	0.01	0.03	0.01	0.01	0.03
		<	<		<	<	
i-18 : 0	*17.69*†	0.01	tr	0.01	0.01	0.01	0.02
		<	<		<	<	
18 : 0	*18.00*∗	0.06	0.07	0.07	0.03	0.03	0.10
		0.01	*0.01*		*0.01*	<	
19 : 0	*19.00*∗	0.01	0.01	n.d.[h]	0.01	0.01	0.04
		<	<		<	<	
20 : 0	*20.00*∗	0.05	0.05	0.25	0.06	0.07	0.35
		0.02	*0.02*		*0.01*	*0.01*	
20 : 0 Ac		0.03	0.02	0.16	0.03	0.04	0.25
		0.02	<		*0.01*	*0.01*	
21 : 0		0.04	0.01	0.03	0.02	0.02	0.03
		0.01	<		<	<	
22 : 0		0.64	0.46	3.16	1.98	1.64	4.42
		0.07	*0.06*		*0.22*	*0.12*	
22 : 0 Ac		0.11	0.06	1.58	0.21	0.15	3.38
		0.01	*0.01*		*0.03*	*0.01*	

(continued on next page)

Table 4 (*continued*)

C_n	ECL[b]	Autumn Bliss[a]			Malling Jewel[a]		
		Aphid-free[c,d]	+Aphids[c,e]	Tops[f]	Aphid-free[c,d]	+Aphids[c,e]	Tops[f]
23 : 0		0.13	0.12	0.25	0.22	0.19	0.15
		0.03	*0.03*		*0.01*	*0.07*	
24 : 1		0.04	0.05	n.d.	n.d.	0.10	n.d.
		<	<			<	
24 : 0		12.16	12.28	13.70	20.75	21.49	9.38
		0.70	*0.07*		*0.73*	*0.77*	
24 : 0 Ac		1.97	1.68	16.79	2.91	2.25	23.41
		0.16	*0.26*		*0.14*	*0.14*	
25 : 0		1.55	1.39	2.16	1.66	1.78	0.93
		0.09	*0.12*		*0.05*	*0.03*	
26 : 1		2.35	2.34	0.27	3.13	2.61	0.23
		0.03	*0.06*		*0.05*	*0.06*	
26 : 0		45.35	46.76	17.49	38.15	39.15	10.67
		0.61	*1.17*		*0.63*	*0.92*	
26 : 0 Ac		5.00	3.66	20.05	3.40	2.84	23.54
		0.42	*0.69*		*0.43*	*0.09*	
27 : 0		0.99	1.07	2.07	0.96	0.88	0.77
		0.17	*0.03*		*0.11*	*0.14*	
28 : 1		0.34	0.31	n.d.	0.48	0.36	0.08
		0.02	<		*0.04*	*0.02*	
28 : 0		10.81	10.05	4.85	10.57	10.19	4.93
		0.76	*0.11*		*0.81*	*0.53*	
28 : 0 Ac		1.08	1.11	4.99	1.04	0.90	5.12
		0.09	*0.04*		*0.06*	*0.06*	
29 : 0		0.26	0.28	0.23	0.20	0.24	0.11
		0.05	*0.02*		*0.02*	*0.01*	
30 : 1		0.28	0.29	n.d.	0.26	0.23	n.d.
		0.01	*0.01*		<	*0.10*	
30 : 0		7.81	8.42	3.40	7.46	8.10	3.67
		0.27	*0.18*		*0.09*	*0.70*	
30 : 0 Ac		0.44	0.54	1.93	0.39	0.40	1.80
		0.04	*0.12*		*0.07*	*0.07*	
31 : 0		0.10	0.14	0.14	0.04	0.04	n.d.
		0.01	*0.04*		<	*0.01*	
32 : 1		0.94	1.02	0.21	0.49	0.56	0.27
		0.06	*0.11*		*0.04*	*0.02*	
32 : 0		5.22	5.44	2.58	3.67	4.22	2.45
		0.17	*0.29*		*0.27*	*0.19*	
32 : 0 Ac		0.54	0.60	1.08	0.34	0.30	1.49
		0.05	*0.03*		*0.01*	*0.03*	
33 : 0		0.08	0.14	0.05	0.02	0.02	n.d.
		0.02	*0.04*		<	<	
34 : 1		0.29	0.34	0.31	0.17	0.14	n.d.
		0.05	*0.03*		*0.01*	*0.01*	
34 : 0		0.69	0.66	0.89	0.67	0.56	1.41
		0.11	*0.10*		*0.06*	*0.01*	
36 : 1		0.26	0.26	0.15	0.30	0.19	n.d.
		0.11	*0.08*		*0.08*	*0.01*	
36 : 0		0.27	0.26	0.92	0.31	0.20	0.71
		0.11	*0.06*		*0.06*	*0.01*	

[a] Samples were taken from the crown of emerging leaves (tops) and from groups of four leaves immediately below the crown. Values are expressed as a percentage of total free primary alcohols in each extract.

[b] Equivalent Chain Lengths (ECL). Values for *iso*- (*i*-) and unidentified branched- (*br*-) saturated alcohols (†) were derived by interpolation from a graphical plot of log GC retention time for straight-chain *n*-saturated alcohols against their assigned ECL values (∗).

[c] Values are means and standard deviations for three replicates each of four or five plants.

[d] Control plants, not subject to bioassay with *A. idaei*.

[e] Plants subject to bioassay with *A. idaei*.

[f] Combined sample from all plants.

[g] < : Standard deviation < 0.005.

[h] n.d.: Not detected.

Table 5
Distribution of alkanes and alkenes in epicuticular wax from raspberry cultivars, Autumn Bliss and Malling Jewel

C_n	Autumn Bliss[a]			Malling Jewel[a]		
	Aphid-free[b,c]	+Aphids[b,d]	Tops[e]	Aphid-free[b,c]	+Aphids[b,d]	Tops[e]
Alkanes and alkenes						
18 : 0	0.06	0.03	0.05	0.03	0.03	0.04
	0.05	*0.02*		*0.01*	*0.02*	
19 : 0	0.04	0.02	0.03	0.04	0.06	0.03
	0.03	< [f]		*0.02*	*0.01*	
20 : 0	0.04	0.02	0.05	0.05	0.06	0.03
	0.02	<		*0.02*	*0.01*	
21 : 0	0.01	0.01	0.01	0.01	0.01	0.01
	<	<		<	<	
22 : 0	0.12	0.11	0.20	0.27	0.28	0.15
	0.06	*0.01*		*0.05*	*0.05*	
23 : 0	0.40	0.42	0.84	0.86	0.82	1.25
	0.14	*0.06*		*0.02*	*0.15*	
24 : 0	0.39	0.52	0.34	0.73	1.07	0.39
	0.22	*0.19*		*0.50*	*0.98*	
25 : 0	1.27	1.21	3.04	2.81	2.87	4.77
	0.30	*0.12*		*0.32*	*0.20*	
26 : 0	0.25	0.23	0.44	0.42	0.40	0.32
	0.06	*0.04*		<	*0.05*	
27 : 0	5.64	4.78	7.08	6.85	7.11	7.07
	1.25	*0.12*		*0.65*	*0.33*	
28 : 0	0.60	0.59	0.50	0.72	0.85	0.54
	0.15	*0.06*		*0.08*	*0.19*	
29 : 0	42.68	43.42	27.37	42.56	38.15	28.04
	2.42	*0.64*		*2.83*	*1.42*	
30 : 0	1.59	1.37	1.20	1.62	1.64	1.08
	0.49	*0.11*		*0.08*	*0.14*	
10-Me-30 : 0	2.62	3.03	n.d.[g]	n.d.	n.d.	n.d.
	1.43	*0.23*				
31 : 0	28.92	30.59	29.26	26.63	29.34	25.02
	1.07	*1.36*		*0.38*	*0.15*	
32 : 0	0.01	0.79	2.00	1.08	1.07	1.48
	0.71	*0.12*		*0.14*	*0.06*	
10-Me-32 : 0	4.75	5.30	n.d.	n.d.	n.d.	n.d.
	3.68	*0.91*				
33 : 1	n.d.	n.d.	1.21	n.d.	n.d.	1.77
33 : 1	n.d.	n.d.	1.54	n.d.	n.d.	2.57
33 : 0	4.80	3.74	8.17	7.43	7.58	7.72
	1.47	*0.50*		*1.13*	*0.15*	
10-Me-34 : 0	0.18	0.16	n.d.	n.d.	n.d.	n.d.
	0.10	*0.03*				
35 : 1	n.d.	n.d.	1.27	n.d.	n.d.	2.28
35 : 1	n.d.	n.d.	1.57	n.d.	n.d.	2.27
35 : 0	2.33	1.81	4.76	4.18	5.08	4.40
	1.15	*0.29*		*0.43*	*0.34*	
10-Me-36 : 0	0.10	0.12	n.d.	n.d.	n.d.	n.d.
	<	0.01				
37 : 1	n.d.	n.d.	2.83	n.d.	n.d.	3.63
37 : 1	n.d.	n.d.	0.69	n.d.	n.d.	1.23
37 : 0	1.47	1.15	2.94	2.34	2.45	2.47
	0.78	*0.14*		*0.43*	*0.14*	
39 : 1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
39 : 1	n.d.	n.d.	1.34	n.d.	n.d.	0.49
39 : 0	0.71	0.57	1.27	1.38	1.13	0.91
	0.33	*0.05*		*0.21*	*0.25*	

[a] Samples were taken from the crown of emerging leaves (tops) and from groups of four leaves immediately below the crown. Values are expressed as a percentage of total alkanes.
[b] Values are means and standard deviations for three replicates each of four or five plants.
[c] Control plants, not subject to bioassay with *A. idaei*.
[d] Plants subject to bioassay with *A. idaei*.
[e] Combined sample from all plants.
[f] < : Standard deviation < 0.005.
[g] n.d.: Not detected.

Table 6
Distribution of secondary alcohols, ketones, tocopherols, sterols and amyrins in epicuticular wax from raspberry cultivars, Autumn Bliss and Malling Jewel

	Autumn Bliss[a]			Malling Jewel[a]		
	Aphid-free[b,c]	+ Aphids[b,d]	Tops[e]	Aphid-free[b,c]	+ Aphids[b,d]	Tops[e]
Secondary alcohols						
Nonacosan-15-ol	n.d.[f]	n.d.	n.d.	21.63	51.50	69.85
Nonacosan-14-ol	n.d.	n.d.	n.d.	n.d.	n.d.	30.15
Nonacosan-7-ol	30.06	n.d.	n.d.	11.96	n.d.	n.d.
Nonacosan-5-ol	69.94	100	n.d.	66.41	48.50	n.d.
Ketones						
Nonacosan-15-one	n.d.	n.d.	100	100	100	88.07
Nonacosan-13-one	n.d.	n.d.	n.d.	n.d.	n.d.	5.22
Nonacosan-12-one	n.d.	n.d.	n.d.	n.d.	n.d.	6.71
Tocopherols						
δ-Tocopherol	29.15	30.51	12.56	37.17	37.01	68.76
	1.13	*1.91*		*1.29*	*0.46*	‘
γ-Tocopherol	70.30	69.06	87.44	61.97	61.81	31.24
	1.15	*1.94*		*1.32*	*0.47*	
α-Tocopherol	0.55	0.43	n.d.	0.85	1.18	n.d.
	0.02	*0.03*		*0.03*	*0.02*	
Sterols						
Cholesterol	tr	tr	n.d.	n.d.	n.d.	n.d.
Campesterol	0.70	0.58	n.d.	n.d.	n.d.	n.d.
	0.26	*0.12*				
Stigmasterol	tr	tr	n.d.	n.d.	n.d.	n.d.
β-sitosterol	3.49	2.54	5.97	n.d.	n.d.	56.25
	1.03	*0.61*				
Cycloartenol	95.81	96.88	94.03	100	100	43.75
	0.77	*0.68*				
Amyrins						
β-Amyrin	61.54	67.74	41.67	90.70	94.18	50.07
	–	–		*2.30*	*0.30*	
α-Amyrin	38.46	32.26	58.33	9.30	5.82	49.93
	–	–		*2.30*	*0.30*	

[a] Samples were taken from the crown of emerging leaves (tops) and from groups of four leaves immediately below the crown. Values are expressed as a percentage of each class of compound or as the percentage distribution of individual positional isomers.
[b] Values are means and standard deviations for three replicates each of four or five plants.
[c] Control plants, not subject to bioassay with *A. idaei*.
[d] Plants subject to bioassay with *A. idaei*.
[e] Combined sample from all plants.
[f] n.d.: Not detected.

rated compounds, the acids also included branched saturated and mono unsaturated components. Some of the branched acids were identified as having a single methyl group on the penultimate (*iso*) and antepenultimate (*anteiso*) carbon atoms by comparison of their chromatographic retention with data obtained in our laboratories for free *iso*- and *anteiso*- acids in the epicuticular waxes of various brassica species including kale, swede (Shepherd et al., 1995a, 1995b, 1997; unpublished data) and broccoli (unpublished data). The chromatographic behaviour of all fatty acids detected in raspberry wax, up to C_{20}, is characterised in Table 3 in terms of equivalent chain lengths (ECL) as described previously (Shepherd et al., 1995a). Similarly, comparison of the chromatographic behaviour of the unsaturated acids in terms of ECL values, with published data, and with a fish oil fatty acid methyl ester standard mixture, suggests that the predominant acids are the 14 : 1 (n-5), 16 : 1 (n-7), 17 : 1 (n-9), 18 : 1 (n-9) and 18 : 2 (n-6), and that the minor acids are the 16 : 1 (n-5) and 18 : 1 (n-7) isomers. The position of the double bond is indicated relative to the terminal carbon, rather than the carboxyl carbon as is usually the convention. Thus, for example, 14 : 1 (n-5) is 9*Z*-tetradecenoic (myristoleic) acid, 16 : 1 (n-7) is 9*Z*-hexadecenoic (palmitoleic) acid, 18 : 1 (n-9) is 9*Z*-octadecenoic (oleic) acid and 18 : 2 (n-6) is 9*Z*,12*Z*-octadecadienoic (linoleic) acid. The minor 14 : 1 acid could not be identified from the available data.

Overall, acids were of similar abundance in wax from mature leaves from both raspberry genotypes (Table 2, Fig. 1(a)). However, there were differences

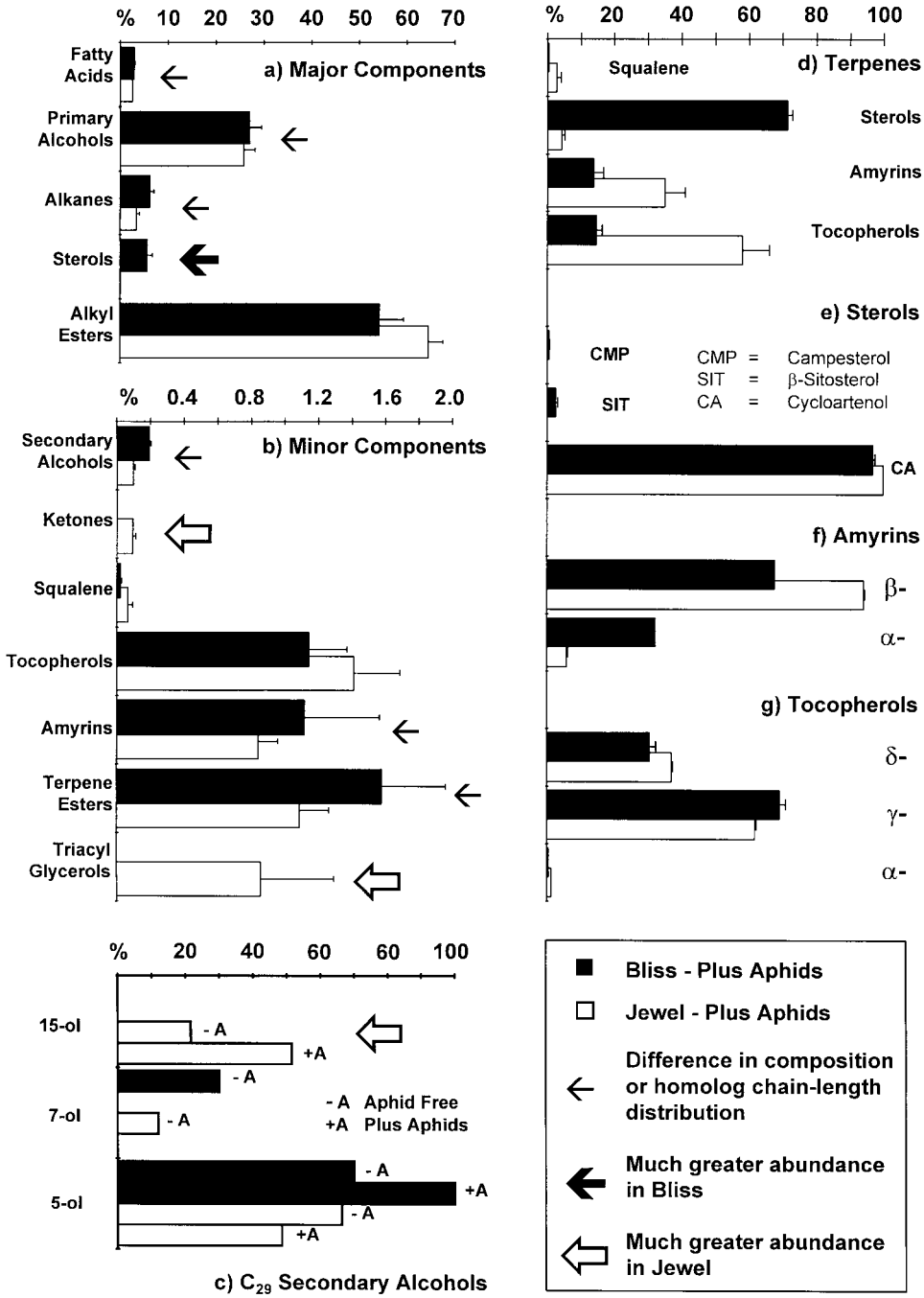

Fig. 1. Distribution of epicuticular wax components from mature biologically active leaves of the raspberry cultivars, Autumn Bliss and Malling Jewel, which had been exposed to raspberry aphid. (a) Major and (b) minor components by compound class; (c) individual C_{29} secondary alcohols; (d) sterols, amyrins and tocopherols as a proportion of total terpenes; and (e) individual sterols, (f) amyrins and (g) tocopherols.

between the genotypes in the distribution of individual acids, and this was most evident for plants exposed to *A. idaei*. The shorter homologues (C_{12}–C_{16}) and the longer homologues (C_{28}, C_{30}) were relatively more abundant in wax from Jewel than Bliss, whereas the mid-range homologues C_{18}–C_{26} were relatively more abundant in wax from Bliss than Jewel (Table 3, Fig. 2(a)). In general, acids were less abundant in wax from emerging leaves of Bliss, in comparison with wax from the more mature leaves, and a shift was observed in the distribution of acid chain lengths from longer (C_{26}–C_{32}) acids towards shorter acids (C_{12}–C_{18}). Conversely, acids were more abundant in wax from immature leaves from Jewel, with no significant differences in the distribution of individual acids between younger and older leaves.

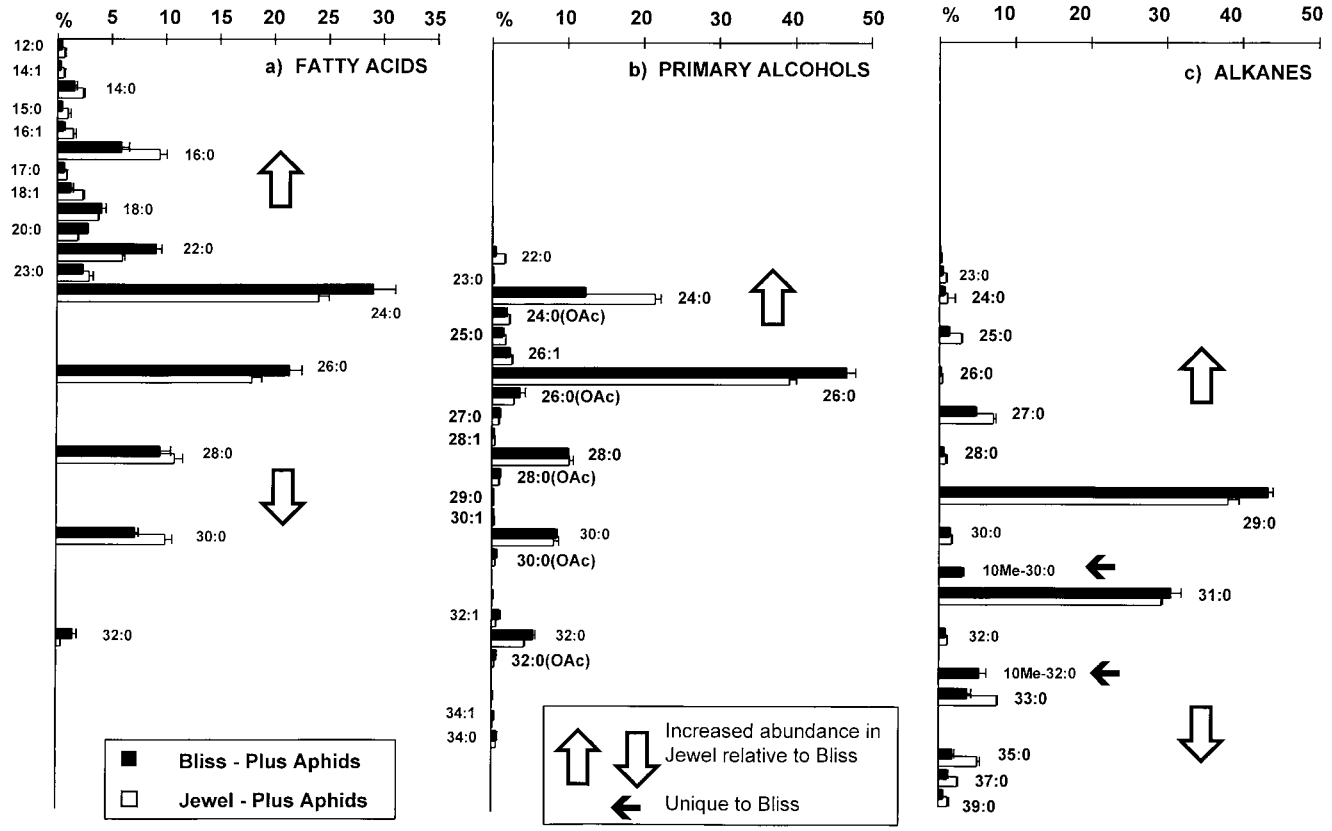

Fig. 2. Distribution of the major homologues of: (a) free fatty acids, (b) free primary alcohols and (c) free alkanes in epicuticular wax from mature biologically active leaves of the raspberry cultivars, Autumn Bliss and Malling Jewel, which had been exposed to raspberry aphid.

2.2.2. Primary alcohols and alcohol acetates

Free primary alcohols (as TMSi ethers) with even carbon numbers predominant were found in the range C_{13}–C_{36}, of which the C_{24}–C_{32} homologues were most abundant (Table 4, Fig. 2(b)). The overall range of homologues was similar to that found for the free acids, although the distribution was different with a maximum at C_{26} rather than C_{24} as found for the acids (Fig. 2(a)). The distribution of alcohols was similar to that of free primary alcohols in rose petal wax (Mladenova et al., 1977) but was wider than that reported for most other leaf waxes and fruit skins (Baker et al., 1979; Bukovac et al., 1979; Ismail et al., 1977; Bianchi, 1995). Several branched alcohols were present, some of which were identified from their chromatographic behaviour as *iso*- compounds (ECL values shown in Table 4), as outlined for the acids (Shepherd et al., 1995a). Mono-unsaturated alcohols (C_{24}–C_{34}) were present in wax from more mature leaves, but were much less abundant, or not detected, in wax from emerging leaves. The major alcohols were also present as acetates, with their abundance relative to the respective unacylated forms falling off with increasing chain length. Acetates were of much greater abundance in wax from emerging leaves where levels were up to twice those of the free alcohols.

In general, overall levels of alcohols (including acetates) in wax from both genotypes were similar (Table 2, Fig. 1(a)). There was, however, a significant difference between the two genotypes in the distribution of individual alcohols and alcohol acetates in wax from the more mature leaves (Table 4, Fig. 2(b)). The C_{22} and C_{24} homologues were relatively more abundant in wax from Jewel than Bliss, and the C_{26}, C_{30} and C_{32} homologues were more abundant in Bliss than jewel.

General levels of alcohols (plus acetates) were reduced by 50% in wax from immature leaves, and in addition to increased abundance of alcohol acetates, there was also a shift towards shorter chain lengths (C_{12}–C_{22}) in the distribution of individual alcohols in wax from the emerging leaves (Table 4).

2.2.3. Alkanes and alkenes

Overall levels of alkanes which were found in the range C_{16}–C_{35}, with odd carbon numbers predominant, were generally similar in wax from more mature leaves of both genotypes (Table 2, Fig. 1(a)). The genotypes did, however, differ in the distribution of individual components (Table 5, Fig. 2(c)). Some 8–9% of the alkanes from Bliss wax were branched isomers with a methyl group at C-10 (10-methyl C_{32} was the major homologue), whereas these isomers were not

detected in wax from Jewel. In addition, Jewel had a wider chain-length distribution with proportionally more *n*-alkanes at shorter (C_{23}, C_{25}, C_{27}) and longer (C_{33}, C_{35}, C_{37}) chain lengths than Bliss (Fig. 2(c)).

Waxes from immature leaves differed from those from the more mature leaves in having appreciable amounts of alkenes, amounting to 10% (Bliss) and 14% (Jewel) of total hydrocarbons. There were also differences in the distribution of chain lengths between the growth stages, immature leaves having less of the C_{29} homologue and more of the shorter C_{23} and C_{25} homologues, and for Bliss, generally more of the longer alkanes in the range C_{33}–C_{39}. The overall alkane distribution with C_{29} predominant is typical of plant leaf waxes (Bianchi, 1995), however, the presence of 10-methyl branched isomers is unusual. The most common branched alkanes in leaf waxes have a methyl group at C-2 (*iso*-) or C-3 (*anteiso*-), or for internally branched compounds, at C-9, C-11, C-13 and C-15 (Holloway, Brown, Baker & Macey, 1977; Bianchi, 1995).

2.2.4. Secondary alcohols and ketones

Secondary alcohols (as TMSi ethers) which were generally more abundant in wax from Bliss than Jewel (Table 2, Fig. 1(b)), consisted of two distinct groups of compounds, the 5- and 7-hydroxy isomers, and the 14- and 15-hydroxy isomers (Table 6, Fig. 1(c)). All four isomers were detected in wax from Jewel, whereas only the former group, the 5- and 7-hydroxy isomers, were detected in wax from Bliss. The 5-hydroxy isomer was generally the most abundant compound in wax from the more mature leaves of both genotypes, whereas neither the 5- or 7-hydroxy isomers were detected in wax from immature leaves of Jewel, in which the 15-hydroxy isomer was predominant. Secondary alcohols were not detected in wax from immature leaves of Bliss. These substitution patterns are different to those found for secondary alcohols in leaf, stem and fruit wax of most plant species studied including other members of the Rosaceae (Holloway & Baker, 1970; Holloway et al., 1976; Ismail et al., 1977; Franich, Wells & Holland, 1978; Baker & Hunt, 1979; Bianchi, 1995; Gaydou, Bombarda, Faure & Wollenweber, 1995), where the hydroxyl group is normally at C-10, C-11 or C-12. Similar positional isomers are, however, found in wax from *Brassica* leaves where substitution is mainly at C-13, C-14, C-15 and C-16 (Netting, Macey & Barber, 1972; Holloway et al., 1976, 1977; Shepherd et al., 1995b) and rose petals where substitution is at C-4, C-5 and C-6 (Mladenova et al., 1977).

Ketones (C_{29}) were detected in the wax from Jewel at both developmental stages, and from emerging leaves of Bliss, however, they were not detected in wax from mature leaves of Bliss (Table 2, Fig. 1(b)). In all cases, the symmetrical positional isomer was predominant (Table 6) which mirrored the position of substitution in the equivalent secondary alcohol, as is usually the case for cuticular wax ketones. No evidence was found for ketones equivalent to the C-5 and C-7 secondary alcohols. The absence of both the 15-hydroxy secondary alcohol and the corresponding ketone from the more mature leaves of Bliss may be indicative of a reduction in the activity of the enzyme systems, whereby these compounds are normally synthesised from the C_{29} alkane.

2.2.5. Terpenes

Several different types of terpenoid compound were found in wax from both genotypes. Overall, levels of these compounds which included squalene, sterols, tocopherols and amyrins were greater in Bliss than Jewel (Table 2, Fig. 1(a), (b) and (d)).

2.2.5.1. Squalene. Squalene, the only acyclic terpenoid compound detected in the wax, was found in all samples, analysed (Table 2, Fig. 1(b) and (d)) with no significant differences between the two genotypes. Squalene has been reported in the epicuticular wax of grapefruit (Nordby & McDonald, 1990, 1991) and raspberry (Robertson et al., 1991).

2.2.5.2. Sterols. The plant sterol precursor cycloartenol was the most abundant sterol found in the wax, with lesser amounts of β-sitosterol and campesterol (all as TMSi ethers) (Table 6, Fig. 1(a), (d) and (e)). Cholesterol and stigmasterol were also detected in trace amounts. Similar sterols have been reported in leaf and flower petal waxes from other members of the Rosaceae (Mladenova et al., 1977; Baker et al., 1979; Bukovac et al., 1979), with the exception of cycloartenol. Sterol levels were significantly lower in Jewel than Bliss. Cycloartenol was the only sterol detected in mature leaves of Jewel at approximately 2% of the levels found for Bliss (Tables 2 and 6, Fig. 1(a), (d) and (e)). Cycloartenol was also present esterified to long-chain fatty acids, and details of this are given in the following paper. The low levels of sterols in wax from Jewel may be indicative of reduced activity of enzyme systems involved in the squalene–cycloartenol–sterol transformation, whereas transformation of squalene to amyrins appears to proceed with equal facility in both Bliss and Jewel. The high levels of cycloartenol relative to the other plant sterols in wax from Bliss may also indicate reduced transformation of cycloartenol to sterols, since cycloartenol is not usually found as a component of cuticular lipid sterol fractions.

Levels of sterols were considerably reduced in wax from younger leaves, and this is in line with studies with maize where sterols levels increased with maturation (Avato, Bianchi & Salamini, 1987).

2.2.5.3. Amyrins. Total levels of amyrins (as TMSi ethers) were similar within the wax of both raspberry genotypes, and amyrin levels were lower in wax from emerging leaves than from more mature leaves (Table 2, Fig. 1(b)). However, the genotypes differed in the proportions of α- and β-amyrin (Table 6, Fig. 1(f)). There was relatively more α-amyrin and less β-amyrin in wax from mature leaves of Bliss (α : β ratio 4 : 6–3 : 7) than Jewel (α : β ratio 1 : 9–1 : 20), and also in wax from immature leaves (Bliss, α : β ratio 6 : 4; Jewel, α : β ratio 1 : 1). The amyrins which are among the most widely distributed triterpenes within plant waxes (Bianchi, 1995) and had previously been identified in raspberry wax (Robertson et al., 1991) were also present in the wax esterified to fatty acids, and details are given in the following paper.

2.2.5.4. Tocopherols. Three members of this class of compound, δ- and γ- and α-tocopherol, were found in the wax (as TMSi ethers), and of these, γ- and δ-tocopherol were most abundant (Table 6, Fig. 1(g)). The δ- and γ-tocopherols were found previously in raspberry wax (Robertson et al., 1991), however, tocopherols are not usually found in plant cuticular waxes, and they have not been reported from other members of the Rosaceae. Overall tocopherol levels were similar for mature leaves from both Bliss and Jewel (Table 2, Fig. 1(b)), although there was relatively more δ-tocopherol and less α-tocopherol in Jewel than Bliss (Table 6, Fig. 1(g)). Tocopherols were less abundant in wax from emerging leaves, where γ-tocopherol was most abundant in wax from Bliss, similar to the pattern for mature leaves, whereas δ-tocopherol was more abundant in Jewel, the reverse of the distribution for the more mature leaves.

2.2.6. Esters

Fully saturated long chain esters were detected in the range C_{36}–C_{54} and these made up the largest proportion of the wax (Table 2, Fig. 1(a)). Esters were of slightly greater abundance in wax from mature leaves of Jewel than from Bliss. Each individual chain length included a number of positional isomers which differed in the length of the esterified acids and alcohols, and full details of these are given in the following paper.

2.3. Role of epicuticular wax as a determinant of insect behaviour

Selection of host plants by the aphidae involves a sequence of specific behavioural events: (a) attraction in response to visual and olfactory cues; (b) testing of the physiochemical characteristics of the leaf surface including probing by stylet; (c) penetration to the phloem tissues and (d) testing of the phloem content (Klingauf, 1987). Feeding and reproduction follow selection of a suitable host. In the absence of the appropriate stimuli, the sequence may be interrupted at any stage, and characteristic behaviour on non-host plants includes increased periods of walking relative to probing, and ultimately the departure of the insect. Behavioural analysis indicates that for *A. idaei*, step (b) is the principal phase of host selection.

During our investigation of *R. idaeus*, the maximum degree of biological activity, expressed by settling and reproduction of *A. idaei*, was observed in the four more mature leaves in the mid-foliar regions. Consequently, consideration of the possible role of wax coverage and composition in conferring resistance or susceptibility to *A. idaei* was centred on the mid-foliar region.

Favourable leaf surface characteristics for successful colonisation by aphids include good surface adhesion and minimisation of physical impediments to movement, probing and stylet penetration. These traits are often associated with glossy (glabrous) phenotypes which usually have reduced coverage of wax, reduced complexity of cuticular wax microstructure and altered chemical composition when compared to the normal waxy (glaucous) phenotypes (Eigenbrode & Espelie, 1995). Increased surface wax levels have been correlated with resistance of cabbage (*Brassica oleracea* L.) to the aphid, *Brevicoryne brassicae* L., of sorghum (*Sorghum bicolor* L.) to the green bug *Schizaphis graminum* (Rondani), of winter wheat (*Triticum aestivum* L.) to the English grain aphid *Sitobion avenae* (F.) (Thompson, 1963; Peiretti, Amini, Weibel, Starks & McNew, 1980; Starks & Weibel, 1981; Lowe, Murphy & Parker, 1986). The general similarity of overall wax coverage on raspberry genotypes Bliss and Jewel would tend to rule out any direct correlation with resistance/susceptibility to raspberry aphid. However, the preference of aphids for older leaves of Jewel may be related to lower wax coverage on these leaves relative to the younger emerging leaves. This type of preference has previously been shown by spotted alfalfa aphids, *Therioaphis maculata* (Buckton), in the foliar canopy of alfalfa (*Medicago sativa* L.) (Bergman, Dillwith, Zarrabi & Berberet, 1991a).

Correlations made between resistance/susceptibility to herbivores and differences in wax composition, along with studies of the biological activity of isolated wax extracts and wax fractions, show that specific wax components may be involved in plant/insect interactions. Each of the major classes of wax component has shown potential for activity, and this may be related to their general abundance in the wax and also to the distribution of individual compounds, including homologues and positional isomers (Woodhead & Chapman, 1986; Eigenbrode & Espelie, 1995).

2.3.1. Fatty acids

The settling of alate green peach aphids, *Myzus persicae* (Sulzer) on hosts was deterred by fatty acids of short chain length (C_8–C_{13}), but was stimulated by fatty acids of chain length greater than C_{16} (Greenway, Griffiths & Lloyd, 1978; Sherwood, Greenway & Griffiths, 1981). Application of dodecanoic acid (C_{12}) to crops reduced settling and crop damage by *M. persicae* (Phelan & Miller, 1982; Herbach, 1987). In our study with raspberry and *A. idaei*, susceptibility was possibly associated with the increased levels of shorter (C_{12}–C_{16}) and longer (C_{28} and C_{30}) acids in Jewel and resistance with the higher levels of mid-length acids (C_{18}–C_{22}) in Bliss (Fig. 2(a)).

2.3.2. Primary alcohols

Primary alcohols may be both stimulants and deterrents, and chain length may again be important. Cottonwood beetles *Chrysomela scripta* (Fabr.), were stimulated to feed by *n*-alcohols (C_{22}, C_{24}, C_{26}, C_{28}, C_{30}) from a beetle-preferring poplar clone (*Populus deltoides* Marshall × *Populus nigra* L.) (Lin, Binder & Hart, 1998). Resistance to tobacco budworm, *Heliothos virescens* (F.), was associated with high levels of docosanol (C_{22}) in wax of host tobacco (Johnson & Severson, 1984), whereas feeding of the larvae of silkworm, *Bombyx mori* L., was stimulated by hexacosanol (C_{26}) and octacosanol (C_{28}) (Mori, 1982). However, for some insects, longer alcohols were associated with resistance. High levels of hexacosanol (C_{26}) in cabbage wax and triacontanol (C_{30}) in alfalfa wax were related, respectively, with resistance to larvae of the diamondback moth, *Plutella xylostella* L., and reduced feeding of the spotted alfalfa aphid, *T. maculata*, (Eigenbrode et al., 1991; Bergman, Dillwith, Zarrabi, Caddel & Berberet, 1991b). In our study, resistance of Bliss to *A. idaea* may have been associated with the greater abundance of the longer homologues (C_{26}, C_{32}) and susceptibility of Jewel with the greater abundance of the shorter homologues (C_{22} and C_{24}) (Fig. 2(b)).

2.3.3. Alkanes

Increased alkane abundance is usually stimulatory, although the chain-length distribution is also important. Shorter *n*-alkanes (C_{19}, C_{21}, C_{23}) from sorghum, but not the longer homologues deterred feeding by *Locusta migratoria* L. (Woodhead, 1983). Alkanes from the host *Vicia faba* L. (main components C_{27}, C_{29}, C_{31} C_{33}) promoted probing and feeding by the pea aphid *Acyrthosiphon pisum* (Harris), in contrast with alkanes from non-host *Brassica* spp., where the chain length distribution was much narrower (mainly C_{29}) (Klingauf et al., 1971, 1978; Klingauf, 1987). Similarly, in our investigation, the wider distribution of alkane chain lengths found for susceptible Jewel in comparison with resistant Bliss may be a factor in the susceptibility of raspberry to *A. idaei* (Fig. 2(c)). The presence of branched alkanes in wax from Bliss may also be a deterrent to *A. idaei*, perhaps by producing an undesirable change in the morphology of the wax surface.

2.3.4. Secondary alcohols and ketones

Plant resistance to certain insects including *S. avenae* and *P. xylostella* has been related to lower levels of secondary alcohols, ketones, diketones and hydroxy ketones in waxes from glossy phenotypes of wheat and cabbage (Lowe et al., 1985; Eigenbrode et al., 1991). Secondary alcohols and ketones were only found as minor wax components during our study of raspberry and *A. idaei*. However, the apparent absence of both the symmetrical C_{29} secondary alcohol (Fig. 1(c)) and the equivalent C_{29} ketone (Fig. 1(b)) from wax of the aphid-resistant genotype Bliss, and their presence in wax from the susceptible genotype Jewel, may be of significance in relation to the behaviour of *A. idaei* on these plants (Table 6).

2.3.5. Triterpenoids

Higher levels of the triterpenols α- and β-amyrin in leaf waxes of cabbage, azalea (*Rhododendron* spp.) and sorghum have been associated with resistance to *P. xylostella*, azalea lace bug, *Stephanitis pyriodes* (Scott), and various aphids; these triterpenols also inhibited feeding by *L. migratora* when added to wheat flour (Heupel, 1985; Eigenbrode et al., 1991; Eigenbrode & Espelie, 1995). Of the other classes of terpene found in leaf waxes, β-sitosterol was shown to stimulate feeding by *B. mori* (Nayar & Fraenkel, 1962; Hamamura, 1970) and α-tocopherylquinone from poplar was shown to stimulate feeding by *C. scripta* (Lin et al., 1998). In our study, there was no correlation between general levels of amyrins and tocopherols and resistance to *A. idaei*, although there were differences in the distribution of individual members of each class (Table 6, Fig. 1(f) and (g)). The comparative effects of the different tocopherols and amyrins on insect behaviour are unknown. However, the higher abundance of α-amyrin in wax from resistant raspberry plants (Table 6, Fig. 1(f)) may be of significance, since α-amyryl palmitate isolated from suberin wax of the sandal tree, *Santalum album* L. is known to affect development of several lepidoptera species (Shankaranaryana, Ayyar & Krishna Rao, 1980). Resistance to *A. idaei* may be associated with the much higher levels of sterols, particularly cycloartenol, found in wax from the resistant genotype Bliss (Table 6, Fig. 1(a), (d) and (e)). We had previously identified cycloartenol and β-amyrin as possible resistance factors during an earlier study, based on multi-component discriminant analysis of

part of the wax from several raspberry cultivars (Robertson et al., 1991).

2.3.6. Esters

In a number of plant species, the abundance of long chain aliphatic esters in the epicuticular wax has been related to insect resistance. This class of compound, along with lesser amounts of terpene esters, constituted the major part of the raspberry waxes studied during our investigation. Their involvement in the interaction with *A. idaei* is considered in the following paper.

In numerous studies, the preference shown by insects for compounds of shorter chain-length provides part of an explanation for their non-selection of immature leaves, since in most plant species studied, chain length increases with maturation (Woodhead & Chapman, 1986; Eigenbrode & Espelie, 1995). This is in agreement with our finding that *A. idaei* displayed a non-preference for immature leaves of the susceptible raspberry genotype Jewel. The factors which have been identified as possible characteristics of resistance to *A. idaei* in raspberry are collectively similar to those identified in other studies with various plants and aphids, but may differ from findings with other insects. We have found similar chemical characteristics for resistance/susceptibility to *A. idaei* in waxes extracted from leaves of field-grown mature raspberry canes (2 years old), at a time when they were known to show the appropriate biological activity (Shepherd, Robertson, Griffiths & Birch, unpublished results). This suggests that some of the characteristics may be general, and may be independent of factors such as plant growth conditions and age, which are known to have a significant effect on wax composition.

During our investigation, we found no significant effects on the composition of the wax which could be attributed specifically to aphids, except for the presence of small amounts of an unusual group of triacylglycerols. These were found exclusively on plants of the susceptible genotype Jewel which had been subject to bioassay with *A. idaei*. Details of these compounds and an explanation of their origin are given in the following paper.

The role of leaf surface chemicals in resistance to *A. idaei* requires further investigation, and this continues in our laboratories. In particular, the physiological effects of specific leaf surface chemicals, and the possibility of synergistic interaction between different wax components, remain to be determined. The spatial heterogeneity of leaf surface chemicals is likely to influence the behaviour of *A. idaei*, since the insect feeds on the lower (abaxial) leaf surface, although initial contact and probably some elements of host selection occur on the upper (adaxial) surface. Alkanes are known to stimulate pea aphids *A. pisum* to move to feeding sites on the lower surface of *V. faba* (Klingauf et al., 1978). Compared with the numerous studies of whole leaf wax, there have been relatively few reports based on differential analysis of upper and lower leaf surfaces. Interestingly, however, during a study of ontogenetic variation in the composition of peach leaf wax, sterols and esters were found only on the upper surface and primary alcohols were prevalent on the upper surface, while hydrocarbons and triterpenoid acids were prevalent on the lower surface (Baker et al., 1979). Such clear differentiation between surface chemistries in another member of the Rosaceae is suggestive that a similar situation may pertain to *Rubus* spp.

3. Experimental

3.1. Plant growth and bioassay with aphids

A total of 40 plants each of red raspberry genotypes Autumn Bliss and Malling Jewel were grown to the 8–10 leaf stage in a glasshouse over the period late spring–mid summer. A selected group of 20 plants of Bliss and 18 plants of Jewel (two plants lost to disease) were then subjected to bioassay with the raspberry aphid, *A. idaei*. Three adult apterous aphids from a non-clonal population of *A. idaei* (biotype 1) were placed on one of the mid-position leaves of each plant and over the next eight days aphid numbers on leaves of susceptible plants increased as a result of parthenogenic reproduction to give a mixed population of adults and nympths of various ages. The location and numbers of all aphids remaining on the plants were then recorded. The aphids were then carefully removed using the moistened tip of a size 2 paint brush in order to prevent damage to either the plants or insects. Leaf surface waxes were then extracted.

3.2. Wax collection and analysis

3.2.1. Wax collection

Aphids were found almost exclusively on the four more mature and expanded leaves immediately below the crown of newly emerging and unfolding leaves. Four of these leaves were detached per plant, weighed and then these leaves from four or five plants were successively dipped for 10 s in dichloromethane (400 ml). The resultant extract was filtered through glass microfibre filter paper and the filtrate was warmed gently on a hotplate, evaporating slowly to approximately 20 ml and then evaporated to dryness under a stream of N_2. The resulting waxy solid was stored in a freezer at $-20^{\circ}C$ prior to chemical analysis. Each group of 20 plants provided four replicate samples for both raspberry genotypes. An identical procedure was repeated with the other control groups of 20 plants from both genotypes, not subjected to bioassay with

A. idaei. In addition, the newly emerging leaves from bioassayed and non-bioassayed plants were bulked together and the waxes extracted to provide single bulk samples of wax from immature leaves of both genotypes. Very few aphids were found on either the emerging leaves or on the remaining basal leaves, and the latter were not sampled.

In the case of the more mature leaves, replicate samples 1–3 were analysed by capillary GC and the data was used for quantification of wax components. Replicate sample 2 was analysed by GC–MS for compound identification and quantification, with co-eluting components such as positional isomers quantified by selected ion monitoring. Replicate 4 was retained for archival purposes. The single samples from the emerging leaves were analysed in a similar fashion.

3.2.2. Sample preparation and analysis by GC and GC–MS

Samples (1.4 mg) were prepared by derivatisation with *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and analysed by capillary GC as described previously for brassica waxes (Shepherd et al., 1995a). GC–MS data were acquired on a Hewlett Packard 5989B quadrupole mass spectrometer, with the following operational parameters. Samples in dichloromethane (1.0–1.5 μl) were introduced onto a DB5-MS column (30 m × 0.25 mm i.d., 0.25 μm film thickness) by cold-on-column injection. The oven temperature programme was 50°C isothermal for 3 min, fast ramp at 30°C min^{-1} to 170°C, then increased by 5°C min^{-1} to 325°C, held at 325°C for 30 min, increased by 2°C min^{-1} to 350°C, and finally held at 350°C for 30 min. The pre-filtered carrier gas employed was high purity helium at a flow rate of 1.4 ml min^{-1} and was pressure-programmed in constant flow mode from 10–29 psi. The mass spectrometer was used in EI mode (70 eV, trap current 300 μA) and scanned over the mass range 30–900 amu in 1 s. The source, quadrupole and interface temperatures were 250, 100 and 325°C, respectively. A high energy dynode detector at 10 kV was utilised to amplify ions at higher masses.

3.2.3. Mass spectral ions used in compound identification

3.2.3.1. Fatty acids. As TMSi derivatives. Identified from $[M]^+$ and $[M\text{-}15]^+$ and fragments at *m/z* 117, 129, 132 and 145.

3.2.3.2. Primary alcohols. As TMSi derivatives. Identified from $[M\text{-}15]^+$, and as acetates identified from $[M\text{-}60]^+$.

3.2.3.3. Branched alkanes. Alkanes with a methyl substituent at C-10 were identified from the mass spectral fragmentation products following cleavage either side of the methyl branch (10-methyltriacontane: $m/z = 309$, 155; 10-methyldotriacontane: $m/z = 337$, 155; 10-methyltetratriacontane; $m/z = 365$, 155; 10-methylhexatriacontane $m/z = 393$, 155).

3.2.3.4. Secondary alcohols. Determined from single ion chromatogram (SIC) areas of mass spectral fragmentation products following cleavage α $[C_nH_{2n}(OTMSi)]^+$ to the OTMSi ether group of TMSi derivatives (nonacosan-5-ol: $m/z = 439$, 159; nonacosan-7-ol: $m/z = 411$, 187; nonacosan-14-ol: $m/z = 313$, 285; nonacosan-15-ol: $m/z = 299$).

3.2.3.5. Ketones. Determined from the SIC of fragmentation products following cleavage α $[C_nH_{2n\text{-}1}O]^+$ and β $[C_{n+1}H_{2n+3}O]^+$ to the carbonyl group (C_{29}: nonacosan-15-one: $m/z = 225$, 241; nonacosan-13-one: $m/z = 197$, 213, 253, 269; nonacosan-12-one: $m/z = 183$, 199, 267, 283).

3.2.3.6. Sterols. As TMSi derivatives. Identified by comparison of mass spectra and GC retention times with those of reference standards and with entries in the Wiley Library of 138K mass spectra (PMB format) (cholesterol: $m/z = 458$, 368, 353, 329, 255, 247, 213, 129; campesterol: $m/z = 472$, 457, 382, 367, 343, 261, 255, 129; stigmasterol: $m/z = 484$, 394, 379, 255, 129; β-sitosterol: $m/z = 486$, 396, 381, 357, 329, 303, 255, 129; cycloartenol: $m/z = 498$, 483, 408, 393, 365, 339, 286.

3.2.3.7. Tocopherols. As TMSi derivatives. Identified by comparison of mass spectra and CG retention times with those of reference standards and published data (Slover, Shelley & Burks, 1967) (δ-tocopherol: $m/z = 474$, 249, 209, 208; γ-tocopherol $m/z = 488$, 263, 223, 222; α-tocopherol $m/z = 502$, 277, 237, 236). γ-Tocopherol coeluted with hexacosanyl acetate on GC, but not on analysis by GC–MS, and quantification was derived from the latter data.

3.2.3.8. Amyrins. As TMSi derivatives. Indentity determined by comparison of mass spectra and GC separation with those of published data (Robertson et al., 1991) and with entries in the Wiley Library of 138K mass spectra (PMB format) (α- and β-amyrin: $m/z = 488$, 218, 203, 189). α-Amyrin partially coeluted with cycloartenol on GC and GC–MS (samples from Autumn Bliss) and was quantified from the SIC for the fragment $m/z = 218$.

Acknowledgements

The authors thank Ms. C. Reid of the Mylnefield Research Services Ltd. Lipid Analysis Unit for supply of fatty acid standards and The Scottish Office Agriculture, Environment and Fisheries Department for financial support.

References

Avato, P., Bianchi, G., & Salamini, F. (1987). In P. K. Stumpf, J. B. Mudd, & W. D. Nes, *The metabolism, structure and function of plant lipids* (p. 549). New York: Plenum Press.

Baker, E. A., Bukovac, M. J., & Flore, J. A. (1979). *Phytochemistry*, *18*, 781.

Baker, E. A., & Hunt, G. M. (1979). *Phytochemistry*, *18*, 1059.

Bianchi, G. (1995). In R. J. Hamilton, *Waxes: chemistry, molecular biology and function* (p. 175). Dundee: The Oily Press.

Birch, A. N. E., & Jones, A. T. (1988). *Ann. Appl. Biol*, *113*, 567.

Bergman, D. K., Dillwith, J. W., Zarrabi, A. A., & Berberet, R. C. (1991a). *Environ. Entomol*, *20*, 470.

Bergman, D. K., Dillwith, J. W., Zarrabi, A. A., Caddel, J. L., & Berberet, R. C. (1991b). *Environ. Entomol*, *20*, 781.

Bukovac, M. J., Flore, J. A., & Baker, E. A. (1979). *J. Am. Soc. Hort. Sci*, *104*, 611.

Eigenbrode, S. D., Espelie, K. E., & Shelton, A. M. (1991). *J. Chem. Ecol*, *17*, 1691.

Eigenbrode, S. D., & Espelie, K. E. (1995). *Ann. Rev. Entomol*, *40*, 171.

Franich, R. A., Wells, L. G., & Holland, P. T. (1978). *Phytochemistry*, *17*, 1617.

Gaydou, E. M., Bombarda, I., Faure, R., & Wollenweber, E. (1995). *Phytochemistry*, *40*, 601.

Greenway, A. R., Griffiths, D. C., & Lloyd, S. L. (1978). *Entomol. Exp. Appl*, *24*, 369.

Hamamura, Y. (1970). In D. I. Wood, R. M. Silverstein, & M. Nakajima, *Control of insect behaviour by natural products* (p. 55). New York: Academic Press.

Herbach, E. (1987). *Ann. Appl. Biol*, *111*, 177.

Heupel, R. C. (1985). *Phytochemistry*, *24*, 2929.

Holloway, P. J., & Baker, E. A. (1970). *Ann. Appl. Biol*, *66*, 145.

Holloway, P. J., Jeffree, C. E., & Baker, E. A. (1976). *Phytochemistry*, *15*, 1768.

Holloway, P. J., Brown, G. A., Baker, E. A., & Macey, M. J. K. (1977). *Chem. Phys. Lipids*, *19*, 114.

Ismail, H. M., Brown, G. A., Tucknott, O. G., Holloway, P. J., & Williams, A. A. (1977). *Phytochemistry*, *16*, 769.

Johnson, A. W., & Severson, R. F. (1984). *J. Agric. Entomol*, *1*, 23.

Jones, A. T. (1979). *Ann. Appl. Biol*, *92*, 119.

Jones, A. T. (1986). *Crop Research*, *26*, 127.

Klingauf, F., Nöcker-Wenzel, K., & Klein, W. (1971). *Z. Pflanzenk. Pflanzenschutz*, *78*, 461.

Klingauf, K., Nöcker-Wenzel, K., & Röttger, U. (1978). *Z. Planzenkr. Pflanzeschutz*, *85*, 228.

Klingauf, F. A. (1987). In A. K. Minks, & P. Harrewijn, *Aphids their biology, natural enemies and control*, *vol. A* (p. 209). Amsterdam: Elsevier.

Lin, S., Binder, B. F., & Hart, E. R. (1998). *J. Chem. Ecol*, *24*, 1781.

Lowe, H. J. B., Murphy, G. J. P., & Parker, M. L. (1985). *Ann. Appl. Biol*, *106*, 550.

Mladenova, K., Stoianova-Ivanova, B., & Daskalov, R. M. (1976). *Phytochemistry*, *15*, 419.

Mladenova, K., Stoianova-Ivanova, B., & Camaggi, C. M. (1977). *Phytochemistry*, *16*, 269.

Mori, M. (1982). *J. Insect Physiol*, *11*, 969.

Nayar, J. K., & Fraenkel, G. (1962). *J. Insect Physiol*, *8*, 505.

Netting, A. G., Macey, M. J. K., & Barber, N. H. (1972). *Phytochemistry*, *11*, 579.

Nordby, H. E., & McDonald, R. E. (1990). *Lipids*, *25*, 807.

Nordby, H. E., & McDonald, R. E. (1991). *J. Agric. Food Chem*, *39*, 957.

Peiretti, R. A., Amini, I., Weibel, D. E., Starks, K. J., & McNew, R. W. (1980). *Crop Sci*, *20*, 173.

Phelan, P. L., & Miller, J. R. (1982). *Entemol. Exp. Appl*, *32*, 46.

Robertson, G. W., Griffiths, D. W., Birch, A. N. E., Jones, A. T., McNicol, J. W., & Hall, J. E. (1991). *Ann. Appl. Biol*, *119*, 443.

Shankaranaryana, K. H., Ayyar, K. S., & Krishna Rao, G. S. (1980). *Phytochemistry*, *19*, 1239.

Shepherd, T., Robertson, G. W., & Griffiths, D. W. (1995a). *Phytochem. Anal*, *6*, 65.

Shepherd, T., Robertson, G. W., Griffiths, D. W., Birch, A. N. E., & Duncan, G. (1995b). *Phytochemistry*, *40*, 407.

Shepherd, T., Robertson, G. W., Griffiths, D. W., & Birch, A. N. E. (1997). *Phytochemistry*, *46*, 83.

Shepherd, T., Robertson, G. W., Griffiths, D. W., & Birch, A. N. E. (1990). *Phytochemistry*, *52*, 1255.

Sherwood, M. H., Greenway, A. R., & Griffiths, D. C. (1981). *Bull. Entomol. Res*, *71*, 133.

Slover, H. T., Shelley, L. M., & Burks, T. L. (1967). *J. Am. Oil Chem. Soc*, *4*, 161.

Starks, K. J., & Weibel, D. E. (1981). *Environ. Entomol*, *10*, 963.

Thompson, K. F. (1963). *Nature*, *198*, 209.

Woodhead, S. (1983). *Physiol. Entomol*, *8*, 345.

Woodhead, S., & Chapman, R. F. (1986). In B Juniper, & T. R. S. Southwood, *Insects and the plant surface* (p. 123). Sevenoaks: Edward Arnold.