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Epicuticular waxes and volatiles from faba bean (*Vicia faba*) flowers

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Abstract

The floral bouquet of faba bean flowers was shown to be a complex mixture of some 27 identifiable compounds. In addition to the ubiquitous mono and sesquiterpenes, the porous-polymer entrained volatiles included a diverse range of phenylpropenoids which together accounted for over 7% of the total. Cinnamyl alcohol was also found to be the most abundant free alcohol in the epicuticular wax of faba bean flowers. Two new classes of epicuticular wax esters consisting of saturated C_{16}, C_{18}, C_{20}, C_{22} and C_{24} fatty acids esterified with the phenylpropenoid, cinnamyl alcohol and with the diterpene, phytol have been identified. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Vicia faba*; Faba beans; Flowers; Volatiles; Phenylpropenoids; Cinnamyl alcohol esters; Phytyl esters

1. Introduction

Flowers attract both pollinators and potential insect pests by a combination of visual, tactile and olfactory cues (Knudsen, Tollsten & Bergström, 1993; Heinrich, 1996; Blight et al., 1997). In the case of the faba bean (*Vicia faba* L.), which is widely grown in Western Europe primarily as a source of plant protein, successful pollination by bees can be an important factor in determining seed yield (Bond & Poulsen, 1983). The scent of faba bean flowers was classified early this century as belonging to the aromatic group, which was characterised as having a sweet odour combined with a spicy quality and whose essential oils commonly contained eugenol and cinnamyl alcohol (Hampton, 1925). However, in a recent study (Sutton, Keegans, Kirk & Morgan, 1992) using polymer entrainment combined with thermal desorption, the only identifiable volatile detected in quantifiable amounts was the monoterpene hydrocarbon (*E*)-β-ocimene. Since this compound has been found to be one of the most common constituents of floral scents (Knudsen et al., 1993), it would appear unlikely that the characteristic odour of field beans could be attributed solely to (*E*)-β-ocimene.

Comparatively little information is available on the biological significance of the waxes located on the surfaces of flowers. It has been suggested (Juniper, 1995) that oils within the flower serve as a glue attaching pollen grains to the insect; protect the pollen grain from water damage and in some flowers may improve water drainage within the flower. Similarly, the detailed chemical composition of waxes present on the surface of flowers has been rarely reported.

The objectives of this study were: (a) to determine whether, using head-space analysis of abscised flowers, compounds other than (*E*)-β-ocimene could be detected in the floral bouquet of field beans; and (b) characterise the major compounds present in faba bean flower epicuticular wax in order to ascertain whether any of the volatile compounds could also be detected on the surface of the flowers.

2. Results and discussion

Polymer entrained volatiles from the head-space of faba bean flowers (cv Maris Bead), were ether eluted

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Table 1
Volatile compounds identified in the headspace of *Vicia faba* (cv Maris Bead) flowers (a is identity based on published mass spectrum; b is identity confirmed by co-elution with authenticated standard; RRI is relative retention index)

Compound	Rt (min)	RRI	Relative concentration (% total area)	Basis of identity
α-Thujene	18.5	941	0.05	a,b
α-Pinene	18.9	953	6.24	a,b
Sabinene	20.8	1014	0.12	a,b
β-Myrcene	21.0	1019	2.49	a,b
(*Z*)-β-Ocimene	22.6	1071	7.83	a,b
(*E*)-β-Ocimene	23.3	1092	67.59	a,b
Benzaldehyde	23.9	1113	0.25	a,b
Linalool	26.7	1202	2.76	a,b
p-Menthatriene	26.9	1208	0.49	a
Benzyl alcohol	27.3	1224	0.04	a,b
p-Allyl anisole	30.0	1311	2.31	a,b
Nerol	31.3	1357	0.82	a,b
Geraniol	32.0	1384	1.62	a,b
p-Propenyl anisole	33.0	1422	1.78	a,b
2-Phenoxyethanol	33.3	1434	0.24	a,b
Neryl acetate	33.9	1454	0.26	a,b
Geranyl acetate	34.4	1474	0.22	a,b
Cinnamyl aldehyde	34.9	1492	0.15	a,b
p-Propenyl phenol	35.1	1500	0.19	a
β-Caryophyllene	35.5	1516	0.78	a,b
Cinnamyl alcohol	35.8	1530	0.77	a,b
Eugenol	36.1	1543	0.66	a,b
O-Methyl eugenol	36.3	1548	0.29	a,b
α-Humulene	36.5	1557	0.10	a,b
α-Farnesene	36.6	1562	0.68	a,b
Unidentified sesquiterpene	37.2	1588	0.39	a
Cinnamyl acetate	38.0	1619	0.86	a,b
Methyl isoeugenol	38.9	1660	0.02	a,b

and analysed by gas chromatography–mass spectrometry (GC–MS). The range of compounds detected are given in Table 1. In addition to (*E*)-β-ocimene, which was previously reported (Sutton et al., 1992) as the sole quantifiable volatile in the head-space of both cut and abscised flowers from this cultivar, a further 27 compounds were detected in quantifiable amounts. The monoterpene hydrocarbons accounted for over 84% of the total volatiles detected with, as expected, (*E*)-β-ocimene predominating. High levels of this compound have been reported previously in other leguminous species such as the sweet pea (Porter, Griffiths, Robertson & Sexton, 1999) and alfalfa (Loper & Berdel, 1978), although its relative concentration appears in both species to be subject to genetic and environmental variation. All other monoterpene hydrocarbons, alcohols and acetates detected in the faba bean flower bouquet were also detected in sweet peas and indeed α-pinene, sabinene, β-myrcene, (*Z*)-β-ocimene and linalool have all been reported as common constituents of floral scents (Knudsen et al., 1993).

Four sesquiterpenes were detected in faba bean flower scent, which together accounted for just under 2% of the total volatiles, with the highest individual concentrations (Table 1) being found for α-farnesene and β-caryophyllene. The unidentified sesquiterpene (Rt = 37.2 min) had an identical mass spectrum and relative retention index (RRI = 1588) to a sesquiterpene, previously found in the floral bouquet of the sweet pea cultivar, Royal Wedding (Porter et al., 1999) and tentatively identified as bicyclosesquiphellandrene.

Aromatic compounds accounted for over 7.5% of the total volatiles. In total 12 such compounds were detected, 9 of which were phenylpropenoids. The predominant compounds in this group were *p*-allyl and *p*-propenyl anisole, which accounted for 4% of the total volatiles. Cinnamyl alcohol and eugenol, which historically have been linked with essential oils from plants producing ‘aromatic’ scents (Hampton, 1925), were each detected at levels exceeding 0.5% of the total volatiles.

The analysis was repeated using an automated thermal desorption unit directly linked to a GC–MS on polymer-entrained flower volatiles from the same faba bean cultivar (Maris Bead) entrained for 8 h. The results (data not shown) confirmed that all the compounds, including the phenylpropenoids, obtained by solvent elution could also be detected at similar relative concentrations by thermal desorption. However, as previously reported (Griffiths, Robertson, Birch &

Brennan, 1999), thermal desorption resulted in the formation of a number of artefacts produced by heat- and metal-catalysed rearrangements of mono- and sesquiterpenes.

In order to determine whether other cultivars also released phenylpropenoids, flowers from a further three faba bean lines, Troy, Tina and Ch170, were entrained for 16 h and analysed by automated thermal desorption. Preliminary experiments with the cultivar, Maris Bead had revealed that prolonged periods (10–16 h) of entrainment resulted in significant break through of the early eluting, volatile compounds, in particular (*E*)-β-ocimene No break through of the less volatile late eluting compounds including the phenylpropenoids was detected. This resulted in an increase in the relative concentration of the less volatile, later eluting constituents. Although this yielded no meaningful data for the relative concentrations in the floral bouquets, the results did confirm that all three cultivars contained the same phenylpropenoids as originally found in Maris Bead. The predominant compounds within this aromatic group were, in all three lines, *p*-allyl and *p*-propenyl anisole. Due to the effects of concentration of the minor constituents, an additional phenylpropenoid, tentatively identified on the basis of its mass spectrum as a trimethoxy-propenyl benzene was also detected.

In order to ascertain whether phenylpropenoids were also present in the epicuticular wax layer of faba bean flowers, dichloromethane extracts of intact flowers were prepared and analysed initially as their trimethylsilyl ether (TMSi) derivatives. The results (Tables 2–4) indicated that the major constituents were alkanes, esters, fatty acids, and alcohols. The most abundant compounds were saturated hydrocarbons (C_{23}–C_{33}), which consisted predominantly of the odd carbon C_{27}, C_{29} and C_{31} alkanes as reported in an earlier study of faba bean petal cuticular lipids (Kolattukudy, Croreau & Brown, 1974). Free fatty acids (C_{14}–C_{26}) accounted for slightly over 8% of the total compounds present in the epicuticular layer with, as expected, the even numbered carbon acids predominating.

Alcohols accounted for a further 6.7% of the total, but in addition to the expected even-carbon numbered fatty alcohols (C_{16}–C_{28}), cinnamyl alcohol was detected. The identity of this phenylpropenoid in underivatised and TMSi derivatised samples were confirmed by comparison of their mass spectra and reten-

Table 2
The acid, alcohol and hydrocarbon composition of wax extracted from *Vicia faba* (cv Maris Bead) flowers

Alcohol	Conc[a]	Acid	Conc[b]	Hydrocarbon	Conc[c]
C16	4.1	C14	2.4	C23	0.9
C18	7.3	C15	1.8	C25	2.4
C20	1.4	C16	36.2	C27	19.2
C22	24.9	C17	0.4	C28	3.5
C24	15.7	C18	16.3	C29	35.3
C26	13.4	C20	9.5	C30	2.5
C28	5.6	C22	13.3	C31	29.2
		C24	15.6	C32	1.2
Cinnamyl	27.6	C26	4.6	C33	5.9
% Total[d]	6.7		8.1		46.5

[a] Concentration expressed as % of total alcohols.
[b] Concentration expressed as % of total acids.
[c] Concentration expressed as % of total hydrocarbons.
[d] Concentration expressed as % all compounds detected (i.e. acids, alcohols, hydrocarbons and esters).

Table 3
The composition of wax esters extracted from *Vicia faba* (cv Maris Bead) flowers

Fatty alcohol based		Phytol based		Cinnamyl alcohol based	
Ester^{n}[a]	Conc[b]	Acid	Conc[c]	Acid	Conc[d]
C32	18.9	C16	2.4	C16	2.6
C34	37.1	C18	8.8	C18	19.4
C36	8.2	C20	54.0	C20	38.1
C38	7.4	C22	24.5	C22	35.4
C40	10.8	C24	10.4	C24	4.4
C42	10.8				
C44	5.0				
C46	1.9				
% Total esters	18.1		18.1		63.8
% Total[e]	7.0		7.0		24.7

[a] *n* is total number of carbon atoms in ester.
[b] Concentration expressed as % of total fatty alcohol esters.
[c] Concentration expressed as % of total phytyl esters.
[d] Concentration expressed as % of total cinnamyl alcohol esters.
[e] Concentration expressed as % all compounds detected (i.e. acids, alcohols, hydrocarbons and esters).

Table 4
The relative concentration (% total fatty alcohol based esters) of individual fatty alcohol based wax esters extracted from *Vicia faba* (cv Maris Bead) flowers

	Acid					
Alcohol	C16	C18	C20	C22	C24	Total
C14	nd	0.4	1.4	nd	nd	1.7
C16	18.7	2.7	1.7	0.8	0.2	24.1
C18	35.2	3.1	0.9	0.3	0.2	39.7
C20	2.5	0.5	0.8	0.3	0.2	4.3
C22	5.4	2.2	4.0	1.7	0.2	13.4
C24	6.8	2.0	1.9	0.3	nd	11.1
C26	3.7	0.7	0.6	nd	nd	5.1
C28	0.5	0.1	nd	nd	nd	0.5
C30	0.2	nd	nd	nd	nd	0.2
Total	72.9	11.7	11.2	3.3	0.8	

tion times with commercially available cinnamyl alcohol and its TMSi derivative. Cinnamyl alcohol was the most abundant free alcohol present in the epicuticular extracts and accounted for over 25% of the total alcohols (Table 2) detected.

Transesterification, followed by TMSi derivatisation of the flower extracts resulted in an increase in the concentrations, relative to that of the alkanes, of both the free fatty acids and fatty alcohols clearly demonstrating the presence of long chain fatty acid-fatty alcohol esters. Utilising a recently developed method for the analysis of intact wax esters (Shepherd, Robertson & Griffiths, 1995), eight peaks were identified in the TMSi derivatised samples as containing various fatty acid-fatty alcohol esters ranging in carbon number from C32 to C46 (Table 3). Different combinations of acids and alcohols may produce coeluting esters with the same total carbon number. The identity and quantity of the individual co-eluting esters within each chromatographic peak were determined by mass spectral analysis based on the characteristic $[RCO_2H_2]^+$ ions derived from the acid moieties by McLafferty rearrangements (Shepherd et al., 1995). These wax esters accounted for 7% of the total and consisted of combinations of even-numbered acids in the range C_{16}–C_{24} with even-numbered alcohols (C_{14}–C_{30}). The predominant acid constituent of these esters was palmitic acid (Table 4), which was present in over 70% of all the esters detected.

Transesterification also yielded a compound identified as the TMSi derivative of the diterpene alcohol, phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol). The identity of this compound was confirmed by comparison of its mass spectrum and retention time with that of a TMSi derivative prepared from commercially available phytol.

An examination of the wax extracts prior to transesterification revealed five peaks with mass spectra containing $[M]^+$ ions consistent with that of C_{16}, C_{18}, C_{20}, C_{22} and C_{24} acid esters of phytol. Each of the five spectra contained fragments with m/z 123, 278 and 296 consistent with the production of the phytol derived ions $[C_9H_{15}]^+$, $[C_{20}H_{38}]^+$ and $[C_{20}H_{40}O]^+$. The acid (RCOOH) moiety present in each phytyl ester was identifiable by the characteristic ions $[RCO]^+$ and $[RCOOH]^+$. No fragment corresponding to the expected McLafferty rearrangement $[RCO_2H_2]^+$ was detected, presumably because of the proximity of the phytol double bond to the ester linkage.

Further evidence of the identity of these compounds was obtained by reacting phytol with synthetically prepared acid chlorides of C_{20} and C_{22} fatty acids, a classic reaction for the preparation of fatty acid esters. GC–MS analysis of the products revealed identical mass spectra and retention times to that obtained for the tentatively identified C_{20} and C_{22} acid phytyl esters present in the epicuticular wax fraction. Phytyl-palmitate has been previously identified in extracts prepared from aerial parts of *Leucas nutans* (Hassan, Burdi & Ahmad, 1991) and from *Pentatropis spiralis* (Rasool, Ahmad & Malik, 1991), but to the best of our knowledge this is the first report of the existence of the higher homologues and of their presence in plant epicuticular waxes.

The relative concentration of cinnamyl alcohol also increased significantly after transesterification, indicating the possible presence of cinnamyl alcohol esters in the epicuticular wax of faba bean flowers. Analysis of the mass spectra from the intact esters revealed the presence of five potential ester peaks with $[M]^+$ ions corresponding to C_{16}, C_{18}, C_{20}, C_{22} and C_{24} acid esters of cinnamyl alcohol. Each mass spectrum had a base peak $m/z = 117$, corresponding to the $[C_9H_9]^+$ ion, also seen as the base peak in the TMSi derivative of cinnamyl alcohol. As with the phytyl esters, the constituent acid was characterised by a major $[RCO]^+$ fragment ion. Cinnamyl alcohol esters containing C_{20} and C_{22} acids were both synthesised via their respective acid chlorides and shown to have identical retention times and characteristic mass spectra to the compounds detected in the epicuticular wax extracts. Methyl and ethyl benzyl alcohols esterified with C22 to C28 acids have been previously reported (Bianchi, 1995), but this would appear to be the first report of cinnamyl alcohol fatty acid esters in epicuticular wax.

The phytyl and cinnamyl esters accounted for 7 and almost 25%, respectively, of the total compounds present in the epicuticular wax of faba bean flowers (Table 3). In contrast to the fatty alcohol esters where compounds esterified with C_{16} acid predominated (Table 4), the phytyl and cinnamyl esters with highest relative concentrations were those containing C_{20} and C_{22} acids (Table 3). The preference of these esters for higher molecular weight fatty acids may indicate that the enzymes involved in their biosynthesis differ from those involved in the synthesis of fatty alcohol esters.

From these studies it emerges that the floral bouquet of faba bean flowers is far more complex than originally postulated (Sutton et al., 1992). In particular a diverse group of phenylpropenoids was identified and although individually present at comparatively low concentrations, the pungent nature of these compounds suggest they may play an important role in the development of the characteristic 'aromatic' aroma associated with faba beans. Additionally, phenylpropenoids in the form of either free cinnamyl alcohol or as fatty acid esters constitute a major proportion of the compounds present in the epicuticular wax of faba bean flowers. Further investigations into the possible role played by such compounds in plant-insect interactions are clearly desirable.

3. Experimental

3.1. Volatile sampling

Flowers from the cultivar Maris Bead were taken from a commercially grown field crop and those from Troy, Tina and Ch170 from glasshouse cultivated plants. In each case 10 flowering racemes consisting 4–6 flowers per raceme were taken and placed into prepierced holes in an aluminium foil covered petri dish containing water. The petri dish was then transferred into a 2 l glass vessel fitted with a flanged multi-port lid. Prior to use, all glassware was baked overnight at 200°. The volatiles were collected in stainless steel columns (89 × 6 mm, Perkin-Elmer, Beaconsfield, UK) containing approximately 250 mg of the porous polymer Tenax TA (2,6-diphenyl-*p*-phenylene oxide), previously conditioned by washing with doubly distilled diethyl ether and flushing with oxygen scrubbed helium for 16 h at 250°. Volatiles were entrained under previously described conditions for periods of 8 or 16 h (Robertson, Griffiths, Macfarlane-Smith & Butcher, 1993).

3.2. Epicuticular wax extracts

Faba bean flowers (150) were taken from a field-grown crop of Maris Bead. The flowers were individually dipped for a maximum of 5 s into 20 ml dichloromethane and, after filtration to remove plant debris, an aliquot (2 ml) was concentrated to 0.1 ml using a stream of gaseous nitrogen and immediately analysed by GC–MS.

3.3. Gas chromatography/mass spectrometry

The solvent eluted volatiles were analysed using a Hewlett Packard 5989 GC–MS fitted with a DB1701 capillary column (60 m × 0.25 mm id, 1.0 μm film; J and W Scientific, Fulsom, California, USA). Details of the analytical conditions were as previously reported (Porter et al., 1999).

The volatiles were thermally desorbed using an automated thermal desorption apparatus (Perkin Elmer, Model ATD50) linked to a Finnigan Masslab Trio 1000 quadrupole GC–MS system fitted with an identical capillary column to that used for the analysis of the solvent eluted volatiles. The desorption parameters, temperature gradient and MS conditions were as in a previous study (Griffiths et al., 1999).

The epicuticular wax extracts were analysed using a Hewlett Packard 5989 GC–MS fitted with a DB-5MS column (25 m × 0.25 mm id with a 0.25 μm film, J and W Scientific, Fulsom, California, USA). The samples (1 μl) were introduced using cold on-column injection. Electronic pressure programming was used to maintain a constant helium carrier gas flow of 1 ml min^{-1}. The oven temperature was initially held at 55° for 5 min, then increased at 4° min^{-1} to 350°. The quadrupole mass spectrometer scanned the range 35–900 amu at a rate of 1 scan s^{-1}. The combined EI/CI source was used in electron impact (EI) mode with an ionisation energy of 70 eV and a trap current of 300 μa. The source and quadrupole analyser temperatures were held at 250 and 100°, respectively. The GC–MS data were processed using the Hewlett Packard G1034 MS Chemstation (DOS series) Software package. Component identification was carried out using the Wiley 138 K mass spectral data base and when available confirmed by comparison of both the retention times and mass spectra with those of authenticated standards.

3.4. Transesterification and TMSi derivatisation

The esters present in the epicuticular wax extracts were broken down into their constituent acid and alcohols and the acids produced simultaneously converted into their methyl esters by transesterification using sodium methoxide (Christie, 1982). TMSi derivatisation of both epicuticular wax extracts and the transesterified wax extracts were prepared using *N*,*O*-*bis* (trimethylsilyl)-trifluoracetamide (Shepherd et al., 1995).

3.5. Synthesis of phytyl and cinnamyl esters

The acid chlorides of eicosanoic (C_{20}) and docosanoic (C_{22}) acids were prepared by adding 0.5 ml oxalyl chloride to 2 mg of acid. After leaving overnight, excess oxalyl chloride was removed in a stream of nitrogen and the remaining acid chloride immediately used in the preparation of the ester.

A dichloromethane solution (0.5 ml) containing the appropriate alcohol at a concentration of 20 mg ml^{-1} was added to the freshly prepared acid chloride and allowed to stand in an ice bath for 1 h. The dichloromethane was removed and the residue re-dissolved in isohexane and washed with water. The organic phase was then passed through an anhydrous Na_2S0_4 column and subsequently loaded onto a Florasil column pre-equilibrated with isohexane. The esters were then eluted with 80:20 isohexane:acetone and, after removal of the solvents, re-dissolved in dichloromethane prior to analysis by GC–MS. The mass spectra of the four synthesised esters are summarised in the following.

Phytyl eicosanoate: MS, EIMS (70 ev), *m*/*z* (rel. int.): 590 [M^+] (3), 380 (4), 312 (6), 296 (9), 295 (7), 278 (39), 179 (9), 137 (17), 123 (88), 111 (38), 95 (76), 82 (66), 71 (67), 57 (97), 43 (100). Phytyl docosanoate: MS, EIMS (70 ev), *m*/*z* (rel. int.): 618 [M^+] (4), 408 (4), 340, (11), 323 (5), 296 (8), 278 (41), 179 (9), 137

(16), 123 (85), 111 (34), 95 (78), 82 (70), 71 (71), 57 (100), 43 (84). Cinnamyl eicosanoate: MS, EIMS (70 ev), *m*/*z* (rel. int.): 428 $[M^+]$ (5), 295 (31), 133 (28), 117 (100), 85 (21), 71 (27), 57 (37), 43 (33). Cinnamyl docosanoate: MS, EIMS (70 ev), *m*/*z* (rel. int.): 456 $[M^+]$ (10), 323 (41), 133 (31), 117 (100), 85, (25), 71 (37), 57 (47), 43 (37).

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