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LIPIDS OF *PINUS HALEPENSIS* POLLEN

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Abstract—The total lipids of *Pinus halepensis* pollen were separated into individual classes of neutral and polar lipids and the components of each class were identified and determined quantitatively. Free fatty acids, waxes and triacylglycerols were found as the main constituents of neutral lipids and phosphatidylcholine and phosphatidylethanolamine of polar lipids. Glycerylether derivatives were detected in neutral and polar lipid fractions. Free and esterified volatile fatty acids were also found in pollen and its neutral lipid fraction.

INTRODUCTION

Lipid materials are localized in various parts of pollen. In the cytoplasm they occur primarily in sperosomes, mainly as triacylglycerols containing linoleic, oleic and palmitic acids [1]. The exine and intine are considered as the primary sites of long chain fatty acids, fatty alcohols and waxes. Terpenes and sterols are probably associated with the cell walls and membranes, but the ease of extraction of sterols from pollen suggests that they are also contained in the cytoplasm [2]. Some individual lipid fractions of pollen from several pine species have been studied in the past (see Discussion), but there is no complete study of all the lipid fractions of the pollen of a single pine species.

Pinus halepensis is a Mediterranean pine species traditionally known for the allergenic properties of its pollen. In this paper we report our findings on the chemical composition of the lipids of the pollen for which, to our knowledge, there is no published report.

RESULTS

The total yield of lipids extracted from four different samples of pollen was found to be $2.5 \pm 0.2\%$ on a dry wt basis. As indicated by phosphorus assays, phospholipids represented *ca* 40% of the crude total lipids (16 ± 2 μg lipid P/mg). TLC analysis indicated the presence of several classes of neutral lipids, phospholipids and glycolipids. Individual lipid classes were isolated by prep. TLC (see Experimental). Bands 1 to 10, in increasing order of R_f value, were extracted, pooled and used for gravimetric analysis (see Table 1), as well as for identification and analysis by HPLC and GC (see below).

Free and bound volatile fatty acids

Fresh pollen was dispersed in water, shaken well and filtered. The filtrate was acidified with phosphoric acid–water, and analysed directly by GC. Volatile acids comprised 0.025% of the pollen and were exclusively acetic (20%) and *iso*-butyric (80%). Volatile fatty acids were also detected in esterified form (mainly in the neutral lipids) by separation into polar and neutral lipids by countercurrent distribution. These two fractions and another aliquot of total lipids were treated with alkali at room temperature. The mixtures were then acidified with phosphoric acid–water and analysed by GC. Volatile fatty acids were detected only in the total and neutral lipid hydrolysates, but not in the polar lipids. Acetic (98.5%) and *iso*-butyric (1.5%) acids comprised 0.06% of the total pollen lipids and acetic (97.56%), *iso*-butyric (1.22%) and valeric (1.22%) acids 0.082% of the neutral lipid fraction.

Polar lipids

The polar lipids in band 1 (PL) isolated by prep. TLC from 40 mg of pollen lipids were analysed by three HPLC methods. Partition chromatography on a polar column [3] permitted the tentative identification of cerebrosides (CER), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylinositol (PI) as main components of pollen polar lipids followed by minor amounts of lysophosphatidylethanolanine (LPE) and lysophosphatidylcholine (LPC). However, in this system PC and sphingomyelin (SM) partially overlap, while CER and PE of the pollen polar lipids were resolved into three and two peaks, respectively. The latter effects are not observed by adsorption chromatography [4, 5]. In this system the resolution of PE, LPE and PI is rather poor, but phosphatidylserine (PS) and LPC are clearly separated. By ion-exchange chromatography [6] the R_ts of PC, SM, LPE and LPC permit the unequivocal identification of these components. In this system SM is resolved into two species according to fatty acid composition [7]. From HPLC it is evident that CER, PC, PE and PI are the main components of the polar lipids of the pollen followed by minor amounts of LPE and LPC (Table 2). Sulphatides (and sterol glycosides) were also detected by analytical TLC of glycolipids.

The quantitative composition of the polar lipids according to HPLC analysis, as well as to the results of

Table 1. Quantitative composition of lipid classes in *P. halepensis* pollen

Prep. TLC band	Lipid class	Gravimetric % total lipids	Gravimetric % of pollen	From GC or HPLC % total lipids	From GC or HPLC % of pollen	From GC or HPLC mmole % of total lipids
1	Phospholipids	37.85	0.946	40.50	1.012	55.81*
2	Glycolipids	8.02	0.200	5.85†	0.147	8.22
3	Monoglycerides	3.95	0.099	3.30	0.082	9.98‡
	Glycerylethers			0.15§	0.004	0.45‖
4	Diacylglycerols	4.18	0.105	—	—	—
	Sterols			1.25	0.031	3.05
5	Fatty alcohols	2.54	0.063	1.08	0.027	4.05
6	Fatty acids	14.56	0.364	15.42	0.385	64.01
7	Triacylglycerols	7.95	0.199	5.56	0.139	19.67**
8	Sterol esters	5.35	0.134	—	—	—
	Fatty acid Me esters			0.97	0.024	3.39
9	Waxes	9.25	0.231	—¶	—	—
10	Hydrocarbons	6.35	0.158	—¶	—	—
	Total	100.0	2.500	74.11	1.851	168.6

*FA of PL estimated as palmitic acid and the 'others' of Table 2 as PC.
†Only CER.
‡FA of MG estimated as palmitic acid.
§GE estimated as chimyl alcohol.
‖From colorimetric determination.
¶Not examined.
**FA of TG estimated as oleic acid.

Table 2. Composition of phospholipids from *P. halepensis* pollen

Phospholipid class	From HPLC %	From P determination (%P)	From P determination (%)
PC	40.06	44.51	44.37
PE	25.02	28.32	26.51
PI	22.80	24.30	27.27
LPC	2.02	2.27	1.51
LPE	1.04	0.60	0.34
Others*	9.06	—	—
Total	100.0	100.0	100.0
% of total lipids	40.50	1.755†	42.06
% of pollen	1.012	0.044	1.051

*Not identified.
†From determination on total phospholipids = 1.826.

phosphorus assays after TLC separation, are shown in Tables 1 and 2. The polar lipids represent *ca* 50% of the total lipids, and contain phospholipids and glycolipids in the proportions *ca* 4:1.

A portion of the polar lipid fraction was submitted to acetolysis and saponification and the derived fatty acids and unsaponifiable products were purified by the dual TLC system. The fatty acids were directly analysed by GC (Table 3) palmitic acid comprising *ca* 96% of the total acids. Minor amounts of decanoic, myristic, stearic and arachidic acids were also detected. Glycerylether determination of the purified unsaponifiable fraction gave 0.00125 μmol glycerylether (GE)/mg of pollen total lipids.

Free fatty acids

The percentage of free fatty acids (band 6 prep. TLC) was the highest among the neutral lipid classes of the pollen samples examined (*ca* 15% of total lipids, Table 1). The acids isolated by prep. TLC were purified by re-chromatography in petrol–Et_2O–HCO_2H (80:20:3) and analysed by GC either as free fatty acids, of after preparation of the fatty acid methyl ester by treatment with BF_3–MeOH. The quantitative results (Table 3) indicate that major components of the pollen free fatty acids were lauric, myristic, palmitic, stearic and oleic acids.

Triacylglycerols

According to the quantitative data, (Table 1) triacylglycerols (TG) are the second most abundant component of the pollen neutral lipids. The TG fraction isolated by prep. TLC (band 7) of pollen total lipids was purified by re-chromatography in petrol–Et_2O–HCO_2H (90:10:1.5) and treated with BF_3–MeOH. The fatty acid methyl esters formed were analysed by GC; myristic, palmitic, stearic, oleic, linoleic and behenic acids were the main fatty acid components (Table 3).

Free sterols

The free sterol–diacylglycerol fraction isolated by prep. TLC (band 4) was re-chromatographed in petrol–

Table 3. Composition (%) of free and esterified fatty acids and free alcohols of *P. halepensis* pollen

Carbon number	Free FAME	Free FA	Free FA as FAME	Esterified FA in TG	Esterified FA in PL	Esterified FA in MG+GE	Free alcohols
< 9:0	+	+	+	+	+	+	—
9:0	T	2.40	1.63	0.12	0.18	T	—
10:0	T	3.70	0.16	1.11	0.23	0.23	T
11:0	T	1.42	1.47	2.73	T	0.62	1.11
11:1	T	1.04	T	0.25	0.24	1.21	—
12:0	10.51	6.48	8.15	0.37	0.45	3.81	1.48
13:0	—	—	—	—	—	—	2.41
14:0	5.46	16.02	18.50	1.36	1.75	10.20	2.22
15:0	1.03	0.71	0.81	0.50	0.24	—	9.72
16:0	27.29	33.20	33.33	16.85	95.74	39.74	5.37
16:1	—	T	T	2.23	—	—	8.05
18:0	15.46	16.21	18.58	10.41	0.68	23.73	—
18:1	12.77	2.27	2.61	36.55	T	2.73	14.63
18:2	2.26	1.10	1.30	14.01	—	3.14	—
18:3	T	0.65	0.73	0.62	—	—	—
20:0	1.55	5.81	1.47	2.48	0.49	14.59	55.01
22:0	33.67	1.62	2.04	4.83	—	—	—
24:0	—	2.92	3.67	2.85	—	—	—
26:0	—	4.41	5.55	2.73	—	—	—
% of total lipids	0.971	15.40	12.27	16.14	12.58	2.597	1.080
% of pollen	0.024	0.385	0.307	0.404	0.314	0.065	0.027

+, Detectable amounts; T, traces.

Et_2O–HCO_2H (40:10:3) and the purified free sterols analysed by GC. β-Sitosterol was the main component (76.4%), followed by ergosterol (12.28%), stigmasterol (7.58%) and minor amounts of cholestanol (1.75%) and cholesterol (1.99%). The free sterol fraction was 1.25% of the total pollen lipids (see also Table 1). The presence of ergosterol was documented by HPLC analysis on a Silica A/10 (Perkin–Elmer) column, along with authenic standards [unpublished data].

Free fatty alcohols

The fatty alcohols isolated by prep. TLC (band 5) were purified by re-chromatography in the system used for free sterols. By GC analysis it was found that 1-pentadecanol, 1-hexadecanol and 1-icosanol were the main saturated components, accompanied by hexadecenol and octedecenol as the sole unsaturated species (see also Tables 1 and 3).

Hydrocarbons, waxes and sterol esters

The lipid fraction isolated from prep. TLC, bands 10, 9 and 8 were purified by re-chromatography in petrol–Et_2O–HCO_2H (90:10:1.5). Portions of the purified hydrocarbon, wax and sterol ester fractions were submitted to strong alkaline hydrolysis. By TLC in petrol–Et_2O–HOAc (70:30:1) it was shown that the saponifiable fractions derived from the wax and sterol ester fractions were fatty acids and the unsaponifiable fractions were free fatty alcohols and free sterols, respectively, and that the hydrocarbons remained non-saponifiable. Quantitative results of gravimetric analysis are given in Table 1. Sterol glycosides were also detected by analytical TLC of glycolipids.

Fatty acid methyl esters

Purification of the lipids of prep. TLC band 8 yielded a fatty acid methyl ester (FAME) fraction. Methyl stearate, methyl palmitate, methyl oleate and methyl behenate were found as the main components of this fraction by GC (see Tables 1 and 3).

Glyceryl ethers

The lipid fraction [monoacylglycerol (MG)–GE] isolated from band 3 by prep. TLC was purified by further TLC. A portion was submitted to acetolysis and saponification, followed by prep. TLC in the dual solvent system for purification of the derived free fatty acids and GE. Lauric myristic, palmitic and stearic acids were found as the main components by GC (Table 3). The purified GE was analysed using a colorimetric assay and gave a value of 0.0045 μmol GE/mg of pollen total lipids (see Table 3). The fatty acid composition of this GE fraction was further investigated by GC and mass spectrometry [unpublished].

DISCUSSION

The overall composition of the pollen lipids of *P. halepensis* was calculated by a combination of the results of gravimetric, GC and HPLC analyses already described

(see Table 1). Existing data on pollen lipids of other pine species allow only a limited comparison. The total lipid content of *P. halepensis* pollen was found to be 2.5% and this is comparable to the contents of 2.73% and 1.80% reported for *P. sabiniana* and *P. radiata*, respectively [8]. Furthermore, the content of total fatty acids (free and esterified) found in this study (1.19% of pollen without fatty acids from diacylglycerols) is quite similar to that of respective contents reported for the pollen lipids [9] of another two pine species (1.25% and 1.33% of pollen).

In other studies of pollen lipids from at least six different pine species [2, 10, 11] it was found that fatty acids with an odd number of carbon atoms were absent, and that unsaturated fatty acids represented more than 50% of the total fatty acids, linolenic acid being the predominant species (up to 30%). However, in our study we found that the lipids of pollen from *P. halepensis* do contain odd carbon numbered fatty acids, 9:0, 11:0 and 15:0, as well as considerable amounts (*ca* 11%) of, C_{20}–C_{26} fatty acids (Table 3) whereas the unsaturated fatty acid content is less than 20%, of which linolenic acid represents an almost negligible part (*ca* 0.4% of total fatty acids). It is noteworthy that palmitic acid represents 96% of the fatty acids of the phospholipid fraction (Table 3), while the TG and other neutral lipid fractions contain a more normal pattern of fatty acid components, of which the unsaturated species (oleate and linoleate) represent a considerable part. Another feature of the pollen samples examined is the high level of free fatty acids (Table 1) as well as the considerable amount of FAME. The latter are probably artefacts formed during treatment with methanol-containing solvents and, in particular, during their evaporation to dryness.

PE, PC and PI accompanied by minor amounts of LPE and LPC were the sole phospholipids identified in *P. halepensis* pollen, but PS, phosphatidylglycerol and *bis*-phosphatidylglycerol were not detected. The latter three phospholipids were also reported in the pollen of *P. ponderosa* [12].

Free and bound sterols were found to occur in *P. pinaster* pollen [2] in the ratio of 1:1.15, while according to the data of Table 1 the ratio of free sterols to sterol esters of *P. halepensis* pollen is *ca* 1:3.5. However, the latter figure is based on gravimetric quantitation of the sterol ester fraction which, most likely, was contaminated with other lipid components of the hydrocarbon (terpene) wax and FAME fraction, as well as with diacyl GE whose presence cannot be excluded since free GE do occur in considerable amounts in the pollen.

The free sterol fraction of *P. halepensis* pollen was found to consist of β-sitosterol (76.4%), ergosterol and stigmasterol together with minor amounts of cholesterol (2%) and cholestanol. The β-sitosterol content of *P. silvestris*, *P. montana* and *P. mugo* pollens was found to be 54%, 65% and 65% of the total sterols, respectively. Campesterol (17%) and cholesterol (8%) were also reported as constituents of *P. montana* pollen [2], while *P. silvestris* was reported [2] to also contain 24-methylenecholesterol (9%). On the other hand, pollen of *P. nigra* was found to contain corticosteroids [13] while low levels of testosterone, epitestosterone and androstenedione were detected in *P. sylvestris* pollen after treatment with β-glycosidase [2] suggesting that these hormones may occur as glycuronides.

Icosanol and octadecenol were the main components of the free fatty alcohol fraction of *P. halepensis* pollen, followed by lower M_r components. Although the fatty alcohol components of the wax fraction were not studied, it is interesting to mention here that a waxy fraction of *P. mugo* pollen was reported to yield mainly tetracosanol, hexacosanol and octacosanol [2].

An important finding of the present study is the identification of GE as well as acetate (and other volatile acids) in free and bound form. GE and acetate are the building blocks of the PAF or AGEPC (plateletactivating factor or acetyl-glyceryl-ether-phosphorylcholine) molecule [14], the allergenic properties of which are qualitatively comparable with those of pollen. Preliminary results of our studies on this subject have already been reported [15].

EXPERIMENTAL

Pollen of *P. halepensis* (*Miller*) was collected in early May from treas at Chalandri (Attica, Greece) in 1980 (0.6 g), 1981 (2.9 g), 1982 (5.4 g) and 1983 (9.5 g). All reagents and solvents were of analytical reagent grade. HPLC solvents were purchased from Rathburn chemicals (U.K). Standards used for HPLC, GC and TLC were obtained from Supelco (U.S.A.) and PolyScience (U.S.A.) and GC supports from Supelco (U.S.A.). Special reagents for TLC visualization were H_2SO_4–H_2O (49:1) for neutral lipids, molybdenum blue reagent [16] for phospholipids and α-naphthol reagent [17] for glycolipids.

Extraction and analysis of lipids. Cell walls of pollen, 2% aq. dispersion, were broken in a 30 ml metallic cylinder (Yeda press, Rehovot) under 60–90 bar pressure of N_2. Lipids were extracted according to a modified method of ref. [18]: 1 *g* of pollen was stirred in 380 ml $CHCl_3$–MeOH–H_2O (1:2:0.8) and filtered. $CHCl_3$ and H_2O were added as required for final ratios of $CHCl_3$–MeOH–H_2O (1:1:0.9). The $CHCl_3$ layer was evapd under vacuum at 30° the residue dried under a steam of N_2 and redissolved in 1–3 ml of $CHCl_3$–MeOH (9:1).

Analytical and prep. TLC of pollen lipids was performed on silica gel G60 0.25 mm and 0.50 mm thickness, respectively. Visualization of spots and bands was effected by exposure to I_2 vapours or by spraying with specific reagents. Analytical TLC identification of neutral lipids was accomplished by two developments (i) Et_2O–HOAc (50:1) up to R_f 0.4 followed by petrol–Et_2O–HOAc (80:20:1), (ii) petrol–Et_2O–HOAc (90:10:1). Analytical TLC identification of phospholipids was accomplished by two developments (i) $CHCl_3$–MeOH–H_2O (13:7:1), (ii) $CHCl_3$–MeOH–conc. NH_3–H_2O (40:60:3:3). Analytical TLC identification of glycolipids was accomplished in $CHCl_3$–MeOH–H_2O (90:10:1). Prep. TLC separation of pollen lipids (40 mg on each chromatoplate) was performed in the dual system Et_2O–HCO_2H (100:6) up to R_f 0.4 followed by petrol–Et_2O–HCO_2H (80:20:3). After prep. TLC individual lipid fractions were extracted from the adsorbent with $CHCl_3$–MeOH–H_2O (1:2:0.8) followed by centrifugation (1500 rpm, 5 min) and conversion of the separated supernatant to 1:1:0.9 by adding the appropriate vols of $CHCl_3$ and H_2O. The $CHCl_3$ layers were evapd under a stream of N_2 and each individual lipid fraction repurified by chromatography (see Results). Polar and neutral lipid fractions were also obtained by a countercurrent distribution method [19]. Strong alkaline hydrolysis [20] was used for deacylation of waxes and sterol esters. A modified method of ref. [21] was used for deacylation and/or dephosphorylation of TG, phosphatides and GE derivatives. The sample was treated with HOAc–Ac_2O (3:2) for 12 hr at 150° in a final concn of 0.04–0.4 mM and then after evapn of solvents under a stream of N_2, with 1 N NaOH–90% EtOH for 2 hr under reflux in a final concn of 0.02–0.1%. Me ester derivative

formation of free and esterified fatty acids [22] was carried out with 14% BF_3–MeOH reagent. Colorimetric assay for free GE and for GE derivatives after acetolysis was carried out according to ref. [23]. The colorimetric assay of ref. [24] was used for the P of phospholipids.

HPLC. Analysis of polar lipids was accomplished in parallel with authentic standards, on stainless steel prepacked columns, by UV detection at 206 nm (1 a.u.f.s.), by injecting 10 μl from a 800 μl soln ($CHCl_3$–MeOH, 1:1) containing the polar lipids from 40 mg pollen lipids. On a 10 μm bonded polar phase column, 30 cm × 4.0 mm i.d. (MicroPack–NH_2, Varian) with solvents A (hexane–*iso*-PrOH–MeOH–H_2O, 55:88:10:15) and B (hexane–*iso*-PrOH, 50:80) at a flow rate of 1.0 ml/min and gradient elution (A, 55% for 10 min; A, 2%/min for 22.5 min; A, 100% for 15 min; equilibration for 10 min) the R_fs of CER, PE, PC + SM, LPE and PI were 6, 16, 22, 25, 28 and 38 min, respectively. On a 5 μm adsorption column, 25 cm × 4.6 mm i.d. (silica B/5, Perkin–Elmer) with solvents A (H_2O) and B (hexane–*iso*-PrOH, 6:8) at a flow rate of 2.0 ml/min and gradient elution (A, 10% for 5 min; A, 1%/min for 10 min; A, 20% for 15 min; equilibration for 10 min) the R_fs of CER, PE, LPE, PI, SM, PC (+SM) and LPC were 4, 6, 7, 7.5, 19.5, 21 and 24 min, respectively. PS standard had a R_f of 14 min. On a 10 μm cation exchange column, 25 cm × 2.6 mm i.d. (Partisil PXS/SCX, Whatman) with the solvent mixture MeCN–MeOH–H_2O (300:150:35) and isocratic flow programing elution (2 ml/min for 2 min; 2–4 ml/min in 4 min; 4 ml/min for 6 min; equilibration for 10 min) the R_fs of PE, LPE, PC, SM and LPC were 2.5, 4.2, 5, 7 and 11 min, respectively. CER and PI standards coeluted with solvent front and PS standard coeluted with PE.

GC. Analysis of free fatty acids was carried out on GP 5% DEGS-PS on Supelcoport; 200° (isothermal); injector and detector, 210°; carrier N_2 (20 ml/min). Analysis of FAME was done on GP 10% SP-2330 on Chromosorb W; oven, injector, detector and carrier as above. Free fatty alcohol analysis was carried out on 10% SR-2330 on Supelcoport; 200° (isothermal); injector and detector 210°; carrier N_2 (30 ml/min). Analysis of volatile fatty acids was carried out on Carbopack C/0.3% Carbowax 20 M/0.1% H_3PO_4; 120° (isothermal); injector and detector, 190°; carrier N_2 (50 ml (min)). Analysis of free (underivatized) sterols was performed on 3% SP-2250 on Supelcoport; 260° (isothermal); injector and detector, 270°; carrier N_2 (60 ml/min).

All analyses were carried out using glass columns 1.8 m × 2.2 mm i.d. and FID.

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