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COMPARATIVE ANALYSIS OF FATTY ACIDS IN POLLEN AND SEED OF RAPESEED

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Abstract—Lipids accounted for 31.7% of the dry wt of mature, viable pollen of rapeseed (*Brassica napus*). External lipids of the pollen coat, specified by the diploid parental genome, and internal cytoplasmic lipids, presumably specified by the haploid pollen genome, were separated by differential extraction. External lipids and paraffins represented 9.8% of pollen dry wt, and contained predominantly linolenic (18:3) with significant amounts of palmitic (16:0), stearic (18:0) and myristic (14:0) acids, as well as paraffins. Internal lipids accounted for 21.9% of pollen dry wt and comprised predominantly 18:3 and 16:0. Mature seeds of *B. napus* comprised 45.0% lipid, with a fatty acid composition different from that of either pollen fraction. Decanoic (10:0), lauric (12:0) and hexadecatrienoic (16:3) acids were detected in pollen but not in seeds. These data are discussed in relation to pollen biochemistry and the potential for pollen selection in rapeseed breeding.

INTRODUCTION

There are two different lipid domains in pollen grains of flowering plants. Firstly, the internal cytoplasmic lipids that are presumed to be products of the haploid genome, and secondly, the external lipids of the pollen coat that are specified by the diploid parental genome and transferred from the tapetal cells late in pollen development [1]. There have been several studies of pollen lipids, but most have provided analyses of only the total fatty acid composition [2]. On extraction with diethyl ether, pollen of various *Brassica* species was found to comprise 8.6–13.1% lipid, on a dry wt basis [3]. However, ether extraction of intact pollen is apparently incomplete. For pollen of loblolly pine, Scott and Strohl [4] reported that ether extraction yielded 1.6% lipid initially, probably representing surface components, followed by a further 7.4%, presumably derived from the cytoplasm, when the grains were fractured.

Nucleic acid hybridization studies in *Tradescantia* and *Zea* have suggested that at least 85% of the genes expressed in the diploid plant may also be expressed in its haploid pollen [5, 6]. If this is so, biochemical markers, such as specific lipid components of interest in oilseed improvement, may be expressed in the pollen and accessible to selection at this stage.

In this report we describe the separation and analysis of external and internal lipid fractions of *B. napus* pollen. Pollen fatty acids were compared with seed fatty acids for two commercial varieties, with a view to establishing whether chemical correlations exist that would allow selection at the gametophyte stage for oil quality in the seed.

RESULTS

Fractionation of external and internal pollen lipids was achieved by first extracting the external lipids of mature pollen with anhydrous acetone, then extracting internal lipids by mechanical fracturing of the pollen grains in chloroform–methanol. This method was developed on consideration of previously published work which showed that extraction of pollen with certain organic solvents was incomplete, and that pollen partially extracted in this way remained of high viability for substantial periods [4,7,8]. This indicates that the plasma membrane, as well as the pollen wall, is a barrier to solvent extraction, and that complete extraction probably only occurs if the grain is fractured. This technique was validated by comparison with histochemical analyses of pollen using lipophilic dyes. In intact grains the dyes stained both the pollen coat and the cytoplasm; after acetone extraction of intact pollen staining was confined to the cytoplasm, indicating that acetone treatment had selectively removed the external lipids of the pollen coat. It is important to note that, using this procedure, it is not possible to identify or prevent minor contamination of the 'external' fraction that may have occurred through partial removal of internal lipids upon initial extraction of the pollen in acetone. However, there should be no cross-contamination of the chloroform–methanol 'internal' extract by external lipids.

Lipids accounted for 45.0% of the seed dry wt for the two cultivars analysed, but not more than 31.7% of the dry wt of mature viable pollen. External lipids accounted for 9.8% pollen dry wt and included significant amounts of paraffins. No paraffins were detected in the internal

lipid fractions, which accounted for 21.9% of pollen dry wt.

Fatty acid compositions of seed and pollen lipids were determined by GC and identifications confirmed by GC-MS with reference to both electron-impact and chemical-ionization spectra. These data are summarized in Table 1. Seeds of the low-erucic acid cultivar, Midas, contained mostly oleic (18:1) and linoleic (18:2) acids, with low levels of eicosenoic (20:1) and erucic (22:1) acids, as reported previously [9]. Seeds of the older cultivar, Norin 16, had significantly lower levels of 18:1, and higher levels of 20:1 and 22:1, which would result in an inferior quality vegetable oil [9]. This negative correlation between 18:1 and 22:1 in rapeseed oils has been described previously [10]. In contrast, the principal components of the pollen lipid fractions were linolenic (18:3) and palmitic (16:0) acids, with little difference between cultivars. Lauric (12:0), decanoic (10:0), hexadecatrienoic (16:3) and myristic (14:0) acids were detected in significant amounts in pollen, but were not detected in the seed. Sequential extraction and analysis of external and internal pollen lipids indicated that these two fractions had very different fatty acid compositions. The external fraction was principally 18:3 with significant amounts of 16:0, stearic (18:0) and 14:0. Internal lipids comprised mainly 18:3 and 16:0.

In the external pollen fraction the paraffins nonacosane (two isomers), heptacosane and pentacosane co-eluted on initial GC analysis with behenic (22:0), 22:1, 20:1 and 18:1 respectively, and constituted 35% of the normalized GC peak areas. These compounds were removed in subsequent analyses by Florisil column chromatography.

DISCUSSION

On a dry wt basis the total lipid and wax content of *B. napus* pollen was only 30% less than that of the seed. However, seed and pollen lipids differed markedly in fatty acid composition. This was apparent not only from the relatively low levels in pollen of some major seed components such as 18:1, 18:2, 20:1 and 22:1, but also from the presence in pollen of 10:0, 12:0, 14:0 and 16:3, which were not detected in the seed. There appeared to be little difference in fatty acid composition between the two different sources of pollen, even though the two varieties differed markedly with respect to fatty acid composition of the seed. Further quantitative analyses would be necessary to establish whether relative amounts of any of the principal fatty acids in pollen can be correlated with relative levels of the major seed fatty acids, and thereby provide a basis for pollen selection for seed oil quality.

The distribution of lipids between the external and

Table 1. Normalized fatty acid composition (%) of total lipid fractions extracted from seed and pollen of the rapeseed cultivars Midas and Norin 16

	Midas			Norin 16		
		Pollen*			Pollen†	
Fatty acid	Seed*	External	Internal	Seed*	External	Internal
10:0	ND	ND	0.2	ND	ND	0.3
12:0	ND	2.1	ND	ND	1.6	ND
14:0	ND	13.0	0.6	ND	17.6	0.7
16:0	4.0	19.8	23.5	3.3	17.9	23.2
18:0	1.7	13.5	0.8	1.4	14.2	0.9
20:0	0.7	6.1	ND	0.8	6.9	ND
22:0	0.3	1.5	0.3	0.5	0.7	0.2
24:0	0.1	0.1	0.5	0.3	0.1	ND
Total saturated	6.8	56.1	25.9	6.3	59.0	25.3
16:1	0.2	ND	ND	0.2	ND	ND
18:1	60.8	5.0	2.1	25.0	3.4	1.9
20:1	2.7	0.6	0.3	11.3	2.4	0.3
22:1	1.7	0.2	0.6	33.6	0.1	0.4
24:1	0.1	0.7	2.8	0.8	0.6	2.5
Monoethylenic total	65.5	6.5	5.8	70.9	6.5	5.1
16:3	ND	2.3	1.1	ND	3.7	1.7
18:2	18.3	5.3	7.3	13.6	4.0	6.0
18:3	8.8	27.5	55.0	7.3	26.1	59.2
20:2	ND	0.2	ND	ND	0.4	ND
Polyethylenic total	27.1	35.3	63.4	20.9	34.2	66.9

*Means of triplicate analyses.
†Means of duplicate analyses.
ND, Not detected.

internal pollen fractions indicated that the pollen cytoplasm contained *ca* twice as much lipid, on a dry wt basis, as the pollen coat. Furthermore, there were some interesting qualitative differences between external and internal lipid fractions, with the external lipids generally comprising more highly saturated fatty acids than the internal lipids. A similar correlation was noted for fatty acids in loblolly pine pollen [4]. These qualitative differences may be the result of the operation of pollen specific genes, such as those observed in *Tradescantia* and *Zea* [6]. A more detailed comparison of fatty acids in developing seed and pollen is necessary to determine the points at which the fatty acid biosynthetic pathways are regulated differently in pollen, tapetum and seeds.

EXPERIMENTAL

Plant material. Field plots of two cultivars of *B. napus* L., Norin 16 and Midas, were grown at the Crops Research Institute, Horsham, Victoria. Pollen and seed samples were collected from the same plots in September and December 1985, respectively. Since the pollen water content, as estimated thermogravimetrically [11], was *ca* 16%, fresh pollen was stored under liquid N_2. When required for analysis, pollen was thawed, stored at room temp. and 95% relative humidity for 30 min and tested for viability using fluorescein diacetate [12]. Samples with less than 70% viable pollen were discarded.

Lipid extraction. Triplicate seed samples were extracted by grinding 43.5 g seed in C_2Cl_4 (50 ml) for 2 min in a ball mill. Total seed oil content was measured using a Foss-Let 15300 instrument (Foss Electric, Denmark). Aliquots were collected for further analysis and solvent was removed by vacuum distillation at 55–65°.

Pollen samples (120–160 mg) were analysed in triplicate (for Midas) or duplicate (for Norin 16). A new method was developed for the sequential extraction of external and internal lipids. Samples were monitored histochemically at different stages of extraction. External pollen coat lipids were extracted by vortexing the pollen sample in anhydrous Me_2CO (3 ml) for 10 min. The extract was separated from the pollen pellet by centrifugation and the pellet was washed again with 3 ml anhydrous Me_2CO. The two extracts were combined, and the solvent removed under a stream of N_2 at room temp. Internal pollen cytoplasmic lipids were then extracted using a modification of the procedure of ref. [13]. The resuspended, partly extracted pollen pellet was stirred vigorously overnight in 3 ml $CHCl_3$–MeOH (2:1), using a Teflon-coated magnetic stirring rod of length just shorter than the diameter of a 10 ml Kimax tube (CIG Medi-Shield, Melbourne). This ensured mechanical fracture of all pollen grains in the suspension. H_2O (1 ml) and hexane (2 ml) were then added and the phases separated by centrifugation. The top phase (MeOH–H_2O) and the remaining hexane–$CHCl_3$ extract was removed after further centrifugation. The pellet was then re-extracted in another 2 ml of hexane and the hexane extracts combined. Solvents were removed under N_2 at room temp.

Histochemistry. The effectiveness of the sequential extraction method for the removal of first external, then internal pollen lipids was monitored histochemically, using the lipophylic dye Oil Red 0 [14, 15] and the fluorochrome Nile Red [16]. Pollen was stained without fixation and mounted in staining medium for microscopy.

Fatty acid analysis. Fatty acids were analysed as Me esters initially by GC and structural confirmation was then obtained using GC-MS. Fatty acid Me esters (FAME) were prepared according to standard procedures [17] using 10–20 mg of pollen lipid and 20–40 mg seed lipid. FAME were analysed using a silanized glass column (1.83 × 0.002 m) packed with 2% SP 2300/3% SP 2310 on Chromosorb WAW 100/120 mesh GP (Supelco); flow rate 12.5 ml/min N_2; temp. programme 185–230° (2.5°/min); oven, injector port and FID at 240°. FAME samples were injected in 0.5–1.0 μl heptane; peak areas were calculated using a computing integrator. Standard FAMEs were prepared from commercially available acids, (Applied Sciences).

An additional step was developed to remove paraffins from external pollen fractions using an activated Florisil column (0.01 × 0.3 m; Adams Scientific, Melbourne). Paraffins were eluted with CH_2Cl_2–MeCN–hexane (125:1:124). Samples were concd using a Kuderna Danish apparatus (100°) and evapd to dryness under N_2 at 90°. GC-MS identification of FAME and paraffins was carried out on a 25 M BP5 fused-silica open-tubular column, (1.0 μm liquid phase coating; SGE, Melbourne). The GC-MS interface temp. was 250°, with the ion source at 200° and manifold heater at 220°. A 70 eV beam was used to obtain EI spectra; isobutane at 0.4 Torr was used as reagent gas for CI spectra.

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